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A study of specificity in mushroom compost.

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A STUDY OF SPECIFICITY IN MUSHROOM COMPOST

SUBMITTED BY

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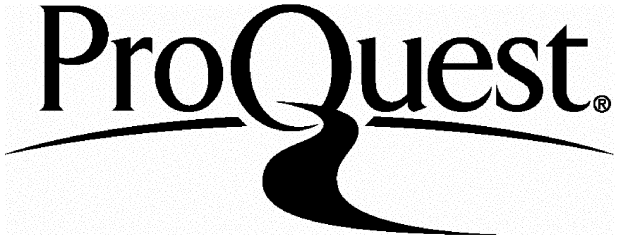

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SUMMARY

Specificity in mushroom compost was demonstrated to be due to nutrient deficiency. Growth of micro-organisms was inhibited by a lack of suitable nutrients which can be induced by microbial leaching. Growth inhibition could not be explained by the presence of antibiotics or volatile compounds. Addition of readily utilisable nutrients resulted in increased microbial activity in compost, this being correlated with reduced growth of A.bisporus.

Specificity was maintained by a number of mechanisms including the production of volatile inhibitors by mushroom mycelium. The products of reactions between phenolic compounds and laccase from A.bisporus to produce inhibitors might also be important in this context. Availability of substrates to micro-organisms and their C:N ratios were important in restricting growth of compost inhabiting fungi. However, A.bisporus was able to synthesise enzymes for substrate degradation at otherwise restrictive C:N ratios. This was attributed to marked biochemical and structural changes within the mycelium. A large number of extracellular enzymes could be induced in A.bisporus suggesting that a wide range of substrates are suitable for degradation.

The selective nature of compost was not explained by differences in inoculum potentials of micro-organisms. When considering the effects of competitive saprophytic ability on specificity it was apparent that substrate degradation was of major importance. A microbiological and microscope study of straw through composting and cropping provided some information on the nature of changes in the substrate with time. During composting a significant increase in microbial biomass occurred on straw, this being markedly reduced during spawn run and corresponding with a reduction in numbers of thermophilic micro-organisms isolated from spawn run compost. Quantitative extraction and chromatographic assay revealed that a major proportion of compost was polysaccharide in nature, the greater proportion of this probably being microbial in origin. This appeared to act as a major nutrient source for A.bisporus during spawn run. Close association with humic acid was suggested to explain lack of degradation of this polysaccharide by other compost inhabitants.

These results suggest that operations aimed at producing express substrates might consider specificity in the light of the mechanisms proposed in the present study and benefit from the knowledge that bacterial extracellular polysaccharides may serve as efficient nutrient sources for mushroom growth.

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1.0 General Introduction

Although the Greeks and Romans were probably familiar with the edible mushroom it was not until the mid fifteenth century that commercial cultivation became popular in France (Ramsbottom 1963). In England commercial cultivation of this fungus has been an established practice for over two hundred years (Atkins 1974). The first English text on the subject (Abercrombie 1779) described the production of mushrooms in glasshouses, barns and in the open. Originally the cultivated mushroom was considered to be Agaricus (Psalliota) campestris Fries. but all mushroom strains used today are derived from A. bisporus (Lang.) Sing. (Singer 1961).

The operations involved in commercial cultivation of mushrooms may be divided into compost preparation, pasteurisation, spawning, crop management, harvesting and disposal of used compost.

Mushroom compost is normally based on wheat straw and horse manure. Wheat straw is used because its cellular structure resists rapid breakdown for long enough to allow aeration of the pile during composting: rye straw degrades too slowly, barley and oat straws degrade too rapidly (Atkins 1974). Compost preparation involves an aerobic thermophilic fermentation of plant and animal residues to produce a substrate suitable for the growth of mushroom mycelium.

Before 1950 composting involved a long aerobic, thermophilic fermentation lasting for up to 4 weeks

(Atkins 1974). This period of preparation was considerably reduced by the introduction of the 2-phase 'Short composting' method (Sinden and Hauser 1950, 1953). Phase - 1 involves an outdoor thermophilic fermentation in stacks of specific dimension for 5-10 days during which time the compost is turned four times, usually on days 0, 2, 5 and 7. Water, and mineral and organic materials may be added during turning. Phase - 2, an indoor process of 3-7 days duration involves a controlled fermentation at 45-55°C with a rise to 60°C for 2-3 hr. at the end to achieve compost pasteurisation. The advantages to be gained through pasteurisation were first noted by Lambert (1938) who recorded the successful elimination of potential pests and pathogens. Phase - 2 is often referred to as peak heating.

After pasteurisation and cooling the compost is spawned. In modern commercial practice this involves inoculating the compost with grain spawn i.e. rye or millet grain colonised by mushroom mycelium. 'Through spawning' is general practice today and involves the mechanical mixing of compost and spawn. Spawned compost is kept at 25°C for two weeks before being covered with a layer of soil or a mixture of peat and chalk (Atkins, 1974). It is in this layer that sporophores are subsequently produced. The environmental conditions and general hygiene measures required during crop production, together with pest and pathogen control, are adequately documented (Anon, 1960; Atkins, 1974).

A common feature of all composting processes is a narrowing of the carbon:nitrogen (C/N) ratio of the substrate. Wheat straw has a C/N ratio of about 70-80:1 and degradation in unsupplemented heaps is slow (Gerrits, Bels-Koning and Muller 1965). By using various materials to increase the organic nitrogen a lower C/N ratio is obtained, this resulting in a more rapid thermogenesis (Gerrits et al 1965). This causes a loss of degradable carbohydrate and a build-up of 'microbial cell substance' (Waksman and Gerretson 1931; Gerrits et al 1965). Although a lowered C/N ratio has been known for some time to promote composting relatively few detailed studies have been carried out on this aspect of mushroom composting. Burrows (1951) and Gerrits et al (1965) have considered the effects of varying initial nitrogen content of compost on C/N ratios and nitrogen contents at spawning. All initial C/N ratio and nitrogen values over a certain range converged to a common point at the end of composting. At spawning the C/N ratios may be about 16-20:1 and the nitrogen content, as determined by Kjeldhal analysis, commonly varies between 1.5% (Burrows 1951; Gerrits et al, 1965) and 2.5% (Atkins 1974). Cropping further reduces the C/N ratio to about 10:1 (Gerrits et al 1965).

During compost preparation a number of different substances may be added as supplements to increase available carbohydrate and organic nitrogen. These are normally added during phase-1 and include urea, nitroform, dried blood, cotton seed meal, chicken manure, molasses,

brewer's grain, hay, cotton seed hulls, malt sprouts, etc. Another approach has involved the supplementation of compost at spawning (Schisler and Sinden 1962a; Delmas and Laborde 1968) and casing (Schisler and Sinden 1962b) with cotton seed meal or soybean meal. At first the resulting yield increases were attributed to additional protein, but were subsequently shown to be due to vegetable oils (Schisler and Sinden 1966; Schisler 1967). Growth of A.bisporus strains in basal medium may be stimulated by lipid supplementation (Wardle and Schisler 1969). Supplementation at casing with ethyl linoleate gave increased mushroom yields which were paralleled using mixtures of safflower oil and linoleic acid (Schisler and Patton 1970). Interestingly strains of A.bisporus not stimulated by lipid supplementation in vitro (Wardle and Schisler 1969) exhibited yield increases when introduced into lipid supplemented compost (Schisler and Patton 1972). Increased sporophore production was attributed to a stimulative effect on mushroom initiation and mycelial growth.

Synthetic compost can be produced without the aid of horse manure. Bulk ingredients such as alfalfa hay, straw and tobacco stems (Waksman and Reneger 1934); hay straw or crushed corn cobs (Yoder and Sinden 1953) and rice straw (Chiang, 1967; Chiou, 1969; Chiou, Cheng and Wang 1972) have been used. Block and Rao (1962) successfully used a number of different types of sawdust, while urban waste has been successfully used for compost preparation by

Franz (1972) and Delmas and Laborde (1972).

Since the introduction of the 2-phase composting method (Sinden and Hauser 1953) other composting techniques have been developed aimed at reducing preparation time, labour and costs. By using a closed slowly rotating container Stoller, Smith and Brown (1937) laid the foundation for the drum composting technique which has been extended by Delmas (1953) and Randle and Hayes (1972). This process has been applied to commercial compost preparation on a limited scale in Australia and the United States of America (Atkins 1974).

Complete composting of materials in one room with a perforated floor and forced air ventilation, combined with a machine to enter the room for turning and spawning operations, was envisaged by Stoller (1952). These thoughts may have prompted development of the recently introduced 3-phase-1 composting technique (Derks 1973) which has also been referred to as 'tunnel composting'.

Several modifications have been devised aimed at dispensing with, or reducing, the requirement to compost substrates. The pioneer work of Till (1962) using autoclaved substrates, spawned in the sterile state and supplemented after spawn run, has been extended by Lemke (1965) and Huhnke and v. Sengbusch (1968). Surprisingly this process has not been adopted commercially, possibly due to the cost of the initial sterilisation, though this has now been replaced by a short steam pasteurisation (Huhnke 1972). Another process of "express substrate preparation" (PES) has been described (Laborde and Delmas, 1969), which

involves shredding and moistening wheat straw, adding organic nitrogen and sugar or vegetable oils before subjecting the mixture to a controlled fermentation in trays in fermentation rooms. Initially a compost temperature of 68°C for 4 hr. is required for compost pasteurisation, followed by incubation at 48°-50°C for 4-7 days depending on the depth of substrate.

A number of modifications of the conventional short composting technique (Sinden and Hauser 1953) have appeared. A 16 day "normal + 75% inactive" composting process (Rasmussen 1962) was aimed at prolonging phase-1 and producing a substrate requiring only a short pasteurisation at filling. Hayes and Randle (1969) attempted to modify phase-1 by adding soluble carbohydrates and thereby dispense with the need for phase-2 which was replaced with a methyl bromide fumigation. However, this process has not gained commercial acceptance because of the hazards involved in using methyl bromide and the increased incidence of pests.

Although Dugger (1905) suggested that the mycelium of A. campestris would grow in unfermented compost, it is now accepted that some form of composting is necessary before successful and rapid colonisation of the substrate by A. bisporus is achieved.

The first study of changes occurring in manure during composting was made by Voelcker (1856). An initial increase in soluble organic matter and nitrogen containing substances was followed by a decline in both these

materials, insoluble organic matter decreased gradually whilst inorganic matter gradually increased.

Falck (1927) distinguished between "hot composting" involving a loss of cellulose, soluble carbohydrates and protein with quantitative accumulation of lignin, and "dissimilative humification" involving a loss of lignin and cellulose. Composting for mushroom cultivation appears to be of the former type (Hebert,1892; Waksman and Diehm,1929; Waksman and Nissen,1932; Gerrits et al, 1965). Losses of cellulose and hemicellulose were accompanied by losses of ammonia, fatty substances, gums and tannins, this being accompanied by an increase in the nitrogen complexes and black materials of compost (Hebert and Heim 1910)

Estimates of cellulose and hemicellulose content of wheat straw vary from more than 60% dry weight (Waksman, Tenney and Diehm 1929) to about 70% (Gerrits, et al 1965) or 80% (Chang 1967), the relative amounts of each in composts depending on concentrations of other additives. Using long composted horse manure losses of cellulose and hemicellulose ranging from 24 to 33% and 11 to 15% respectively (ash free material) were recorded (Waksman and Reneger 1934). When the total quantity of materials in compost was reduced through decomposition by 50-70% a considerable proportion of this was due to carbohydrate loss, the lignin remaining relatively unchanged, and the mineral constituent increasing in proportion to disappearance of organic substances (Waksman and Reneger 1934).

Using the short composting method to produce synthetic composts Gerrits et al (1965) recorded losses in both cellulose and hemicellulose between the start of fermentation and spawning; cellulose was reduced from 36% to 12% and hemicellulose from 22% to 8% on an equal ash basis.

In contrast the lignin in straw from horse manure was relatively resistant to microbial degradation during composting and appeared to accumulate during composting up to the time of spawning, after which time a marked decrease occurred linked with the growth of A. bisporus. (Waksman and Nissen 1931, 1932). With short composted material Gerrits et al (1965) noted that lignin remained practically constant at about 14% (equal ash basis) till spawning, this decreasing to about 7% after mushroom growth.

The nature of the nutrients in compost for mushroom growth has been the subject of numerous studies. Waksman and Nissen (1931) compared compost colonised by mushroom mycelium with uncolonised compost and, after chemical extraction, concluded that the mushroom had utilised lignin and protein as major carbon and nitrogen sources. These findings were reinforced by Waksman and McGrath (1931) and Waksman and Nissen (1932). Results obtained by 'proximate chemical analyses' in these studies also suggested that cellulose might act as a nutrient substrate for the mushroom. However, Treschow (1944) criticised this work since no account had been made of the 'hemicellulose' fraction synthesised as part of the mycelium of A. campestris. In view of this Treschow (1944)

suggested that hemicellulose was a major carbohydrate source for the mushroom in compost.

These early results were confirmed more recently by Gerrits et al (1965). Analysis revealed that of the cellulose and hemicellulose degraded after spawning only 1/3 pentosans (hemicellulose) and 1/8 alpha-cellulose was consumed during spawn run, 2/3 and 7/8 respectively being consumed during cropping. This was taken as an indication that these carbohydrates act as energy sources for the formation of sporophores.

Lignin has long been considered to be a nutrient source for the mushroom (Waksman and Nissen 1931). This early work was subsequently extended and confirmed (Waksman and McGrath, 1931; Waksman and Nissen 1932). Reductions in the lignin content of compost from 22% to 7% were recorded over a long growing period, 250 days (Waksman and Nissen 1932). Gerrits et al (1965) recorded losses of lignin (from 14% to 7%) over a 126 day growing period. Similarly Grabbe (1972) has recorded reductions in compost lignin following mushroom growth.

The nature of the nitrogen supply for mushroom growth in compost has also received much attention. Extensive synthesis of microbial protein from simple nitrogen compounds in manure heaps was claimed by Déhrain (1889) and Hebert (1892). After sequential extraction Waksman and Tenney (1927) and Waksman and Stevens (1930) referred to the difference between total nitrogen and soluble nitrogen as 'crude protein'. It is this protein which has been

implicated as supplying the nitrogen necessary for mushroom growth in compost (Waksman and McGrath 1931; Waksman and Nissen 1932; Gerrits et al 1965). The reasons for this protein not being rapidly degraded in compost were discussed by Waksman and Iyer (1932) who suggested that stabilisation resulted from the formation of Schiff base-type linkages between protein and lignin i.e. ligno-protein complexes. The occurrence of lignin-protein complexes in compost had been suggested much earlier (Dehrain 1889; Hebert 1892) and were considered to be one of the major products of the composting process (Waksman and Nissen 1931). Doubt has been cast on the Schiff base-type linkage complex by Norman (1942) who suggested that only a few peptide linkages would be blocked by the limited number of lignin carbonyl and protein amino groups able to react. However, Lynch and Lynch (1958) demonstrated that protein lignin complexes may be extremely resistant to microbial degradation, and Estermann, Peterson and McLaren (1959) have produced lignin-lysozyme complexes that were resistant to decomposition by chymotrypsin, mixed soil cultures and pure cultures of three bacteria.

According to Gerrits et al (1965) supplementary nitrogen in compost is mainly converted to ammonia during fermentation, this being incorporated into microbial cell components and a 'nitrogen-rich lignin-humus complex'. They suggest that the insoluble nitrogen of this complex is utilised by A.bisporus during mycelial growth in compost.

Similarly, the 'microbial protein' synthesised during composting may act as a nitrogen source (Gerrits et al 1965; Hayes 1972). The role of cell residues other than protein in supplying nitrogen for mushroom growth has not been considered, though Gerrits et al (1965) assumed that thermophilic micro-organisms may be consumed.

The importance of micro-organisms in compost preparation has received a great deal of study. In an early examination of manure Repin (1897) assumed that bacteria utilised all soluble organic matter during compost preparation and in so doing restricted subsequent activity of other micro-organisms. A study of the associative and antagonistic effects of micro-organisms involved in decomposition of plant residues (Waksman and Hutchings 1937) revealed that mixtures of micro-organisms caused the greatest degradation of material. The importance of temperature in this process of decomposition was also studied (Waksman, Cordon and Hulpoi, 1939). Fergus (1964) recorded the numbers of thermophilic fungi and actinomycetes occurring in compost during peak heat, and Laborde, Delmas and d'Hardmere (1968) studied the numbers of thermophilic and mesophilic micro-organisms in compost during preparation without commenting on their significance. Numbers of thermophilic and mesophilic micro-organisms occurring in compost during Phase-1 have been studied (Hayes 1968). He attributed yield increases after fermentation of compost with added sucrose to conservation of nutrients of direct value to the mushroom. The role of cellulolytic thermophilic

micro-organisms in synthesising vitamins and amino acids essential for the growth of A.bisporus was suggested by Stanek (1968). Later he highlighted the possibility that A.bisporus may utilise extra-cellular polysaccharides from bacteria when he demonstrated that the mushroom is able to grow 4-7 times more efficiently on these polymers than on glucose (Stanek 1972).

On the basis of the extraction procedures used Trussov (1917), Waksman (1926) and Jensen (1932) claimed that materials possessing the properties of humic acids occurred in the mycelium of soil fungi, e.g. Polystictus versicolor, Aspergillus niger and a Stachybotrys species. Similarly Thom and Phillips (1932) also claimed to have demonstrated lignin-like materials in the mycelium of soil fungi and suggested that such fungi may contribute to the residual humus in soil. A number of soil fungi are known to synthesise a large number of phenolic compounds in vitro from glucose and asparagine (Lund, Robertson and Whalley 1953; Martin, Richards and Haider 1967). These phenolic compounds may condense into polymers and form nitrogen containing "humic acid" type materials by combination with amino acids or peptides (Haider 1968; Haider and Martin, 1970) or amino sugars (Bondietti, Martin and Haider 1972). These substances are extremely resistant to microbial degradation in soil (Martin et al 1967). Formation of humic substances, possibly of microbial origin, in compost during fermentation has been noted (Haider 1968) but the significance of these materials in mushroom nutrition is considered to be small (Grabbe 1972).

When correctly prepared, peak heated compost is selective for A. bisporus i.e. it possesses a high degree of specificity for mushroom growth. Incorrectly prepared compost may allow the growth of a number of micro-organisms, either competitive or pathogenic towards the mushroom. Competitive fungi may be referred to as 'weed moulds', others which appear as the result of faults in composting technique are termed 'indicator moulds' (Kneebone and Merek 1961; Atkins 1974). A review of ecological control measures required to avoid or reduce contamination of this kind has recently appeared (Sinden 1971).

The object of this investigation was to examine the mechanism of specificity of compost. Any explanation of this phenomenon must account for the failure of many fungi to grow in compost and demonstrate the mechanism which enables A. bisporus to grow freely in this growth medium.

2.0 General Materials and Methods

Unless otherwise stated the materials and methods described below were used throughout this study. Details of more specific materials and methods are described under the appropriate sections.

Compost: Fresh peak heated compost prepared by the short composting method (Sinden and Hauser 1950, 1953) was obtained from Darlington Mushrooms Ltd., Bradford-on-Avon, Wiltshire.

Casing material: A mixture of Irish *Sphagnum* peat and ground limestone (1:1 w/w) was supplied by Darlington Mushrooms Ltd.

Cultivation of *A. bisporus* sporophores:

Peak heated compost was 'through spawned' with 0.2% w/w Darlington 621 strain of *A. bisporus* and packed into plastic bowls (32 x 24 x 12 cm) to within 2-4 cm of the top. After incubation for 14 days at 24°C the compost was covered with a 2-4 cm thick layer of casing material. Incubation was continued at 15-18°C in a humid atmosphere and sporophores were picked after 14-21 days.

Express substrate preparation: Wheat straw was milled to roughly 1.0 cm lengths (milled and supplied by Wrington Vale Mushrooms Ltd., Buxton, Derbyshire), soaked in tap water for 12 hr. and allowed to drain free of excess moisture for 3 hr. Ground calcium carbonate (5% w/w) and peptone (4% w/w) were added with thorough mixing. The substrate was then placed in plastic coated

wire-mesh baskets in humid incubators at 50-55°C for 3 days, followed by a pasteurisation period of 2 hr. at 60°C. During incubation the incubator door was opened for two half hour periods each day to facilitate aeration. These fermented substrates were selective for the growth of A.bisporus, although mycelial growth in them was much reduced compared with traditional compost, being restricted to the production of fine hyphal strands. This method of preparation was based on that of Laborde and Delmas, (1969), and Smith (pers. comm).

Sterilisation: Soil, casing material and compost were sterilised by autoclaving at 15 lb/in^2 for 60 min. on three consecutive days. Components of media for in vitro studies were mixed and exposed to steam for 10 min. prior to autoclaving at 15 lb/in^2 for 15 min.

Cultivation media: Throughout the study a basal medium was used for growth of fungi in vitro. This was amended when required with various carbon and nitrogen sources, usually D-glucose and L-asparagine. The medium was a modification of that used by Levi and Cowling (1969) and contained (g.l^{-1}); KH_2PO4,2.0; Na_2HPO4,1.0; $MgSO4.7H_2O$,0.5; $CaCl_2.2H_2O$,0.002; $FeCl_3.6H_2O$,0.0002; $ZnSO4,7H_2O$,0.0002; $MnSO4,7H_2O$,0.0002; malt extract,0.2. The medium was adjusted to pH 6.5 and was usually used in shallow liquid culture (see below) but where required it was solidified using 12.0g.l^{-1} plain agar (Oxoid No.3).

Inoculation: For routine growth of fungal isolates 0.5 cm. diameter agar discs were cut from the edge of fully colonised plates and placed in the centre of freshly poured plates of 3.0% w/v malt extract agar (Oxoid).

Growth of mycelium in shallow liquid still culture was achieved by using 25 ml medium per 9.0 cm. dia. Petri dish and inoculating as follows. Aerial mycelium from agar cultures was removed with a minimum of attached agar, washed in sterile distilled water and macerated in a sterile, ice cooled 'Potter-type' hand homogeniser. Standard volumes were used to inoculate dishes. Four malt extract agar plates fully colonised by A. bisporus were used to prepared 20 ml inoculation in each experiment.

All isolates of bacteria and actinomycetes obtained from compost were maintained and subcultured on nutrient agar (Oxoid).

Subculturing and inoculation procedures were performed in sterile air in a laminarflow cabinet (Pathfinder, W.H.S. Ltd., Havant).

Incubation: Mesophilic micro-organisms were incubated at 25°C, thermophilic fungi at 45°C, and thermophilic bacteria and actinomycetes at 50-55°C. Incubators were lined with wet tissue paper to maintain humid atmospheres and prevent excessive drying of agar plates.

Mushroom strains: The strains used throughout the study were obtained from the culture collection, University of Bath. Unless otherwise stated Darlington 621 was used.

Isolation of micro-organisms from compost:

Saprophytic bacteria, actinomycetes and fungi were isolated from compost by the dilution plate method (Waksman, 1927; Garrett, 1951) Five grammes of compost were shaken in 50 ml sterile 0.02% w/v agar water (plain agar) and tenfold dilutions prepared. From one or more of these dilutions 1.0 ml samples were removed, placed in Petri dishes and dispersed with 20 ml molten agar cooled to 45-50°C. Malt extract agar with dilutions of 10^{-3} and 10^{-4}, nutrient agar with dilutions of 10^{-6} and 10^{-7}, and half strength nutrient agar with dilutions of 10^{-7} and 10^{-8} were used to isolate fungi, bacteria and actinomycetes respectively. All isolates were stored under sterile paraffin oil. To prevent spread of motile bacteria agar plates were air dried for 10min.

Fungi isolated from compost and used for this study were Absidia sp., Cephaliophthora tropica,* Chaetomium olivaceum,* Coprinus fimentarius,* Fusidium sp., Oedocephalum fimentarium,* Penicillium sp., Stysanus stemonitis,* Sporotrichum thermophile,* Trichoderma sp.,* and Trichothecium roseum. (Those fungi found actively growing in compost at some stage during the study are marked with an asterisk). Isolates of Dactylium dendroides, Polystictus versicolor, Schizophyllum commune and Verticillium malthousei (fungicola) were often included in experiments for

comparative purposes but were not isolated from compost.

Analytical techniques: Those techniques used routinely throughout the study include assays for reducing sugar (Nelson 1944; Somogyi 1952), total soluble carbohydrate (Morris 1948) and α-amino acids (Lee and Takahashi 1966). Standard lines for these assays are given in Appendix 1.

Chemicals: Unless otherwise stated all chemicals were of Standard laboratory grade supplied by Fisons (Cambridge) or British Drug Houses (Poole, Dorset), and all microbiological media were supplied by Oxoid Ltd.

The experiments described below contained three replicates per treatment unless otherwise stated. In cases where such replication proved impractical one replicate was used and any important results checked twice. One large sample of compost was obtained for each experiment and subsampled twice before use.

3.0 Behaviour of fungal spores in compost

3.1 Introduction

The term fungistasis describes the phenomenon whereby viable fungal propagules, not under the influence of endogenous or constitutive dormancy (Sussman and Halvorson 1966), do not germinate in soil in conditions of temperature and moisture favourable for germination (Lockwood 1964). The term may also be applied to conditions, other than temperature and moisture, which retard or terminate hyphal growth in soil (Hsu and Lockwood 1971, Watson and Ford 1972). In this study the term fungistasis will be restricted to the effect of compost on the germination of fungal spores.

The original observations of Simmonds, Sallans and Ledingham (1950), Hessayon (1953) and Chinn (1953) on germination of spores in soil suggested that inhibition might be common. This work was extended to include a wide range of spore types and the results unified in the concept of a 'widespread soil fungistasis' (Dobbs and Hinson 1953). Since that time numerous workers have sought to explain the nature of this phenomenon in soil (for reviews see Lockwood 1964, Watson and Ford 1972).

Fungistasis has been reported in a wide variety of soil types in different geographical locations, and has always been associated with biological activity (Jackson 1958, Lockwood 1964), and may be reversed by heat sterilisation or by the addition of certain levels of nutrients (Dobbs and Hinson 1953).

Two theories have been advanced to explain widespread fungistasis. The diffusible inhibitor theory advocated by Dobbs and Hinson (1953), Jackson (1958), Dobbs and Griffiths (1962) and Lockwood (1964) has received widespread support, but the nature and source of the causal substances remain unknown. This theory seeks to explain soil fungistasis in terms of chemical inhibitors actively produced in soil by micro-organisms. Although changes in populations of antibiotic producing micro-organisms have been observed in amended soils (Weinhold and Bowman 1968; Sneh and Henis 1972) and antibiotics have been demonstrated in soils amended with natural substrates that provide sites of relatively high nutrient status (Grossbard 1948, 1952; Wright 1952; Ragaswami and Ethiraj 1962) all attempts to demonstrate the presence of antibiotic in non-amended soils have been inconclusive. Baker (1968) suggested that this failure to demonstrate the causal factors may be due to inactivation of the substance by adsorption onto clay colloids or humus during extraction.

A second theory attempting to explain fungistasis is that of nutrient deficiency (Ko and Lockwood 1967; Adams, Lewis and Papavizas 1968; Steiner and Lockwood 1969; Hsu and Lockwood 1971). This contends that inhibition of fungal spores in soil is explained by lack of nutrients in the soil essential for germination. Inhibition of spores requiring exogenous nutrients for germination is caused by this general deficiency. That soil is an impoverished medium was suggested by Waksman (1952), Ko and Lockwood (1967) and Gray and

Williams (1971a). Inhibition of spores not requiring exogenous nutrients for germination has been considered to be due mainly to the rapid leaching of metabolites promoted by bacterial activity (Ko and Lockwood, 1967) Subsequently other nutrient independent propagules, e.g. sclerotia have been shown to be inhibited in this way (Adams, Lewis and Papavizas, 1968; Emmatty and Green, 1969; Hsu and Lockwood, 1973). Evidence for the rapid utilisation of energy sources added to soil supports the suggestion that soil acts as a depleting energy sink through potential microbial activity (Rovira 1953; Stevenson 1956; Ko and Lockwood 1967; Bristow and Lockwood 1973).

Though antibiotic production and nutrient deficiency have been invoked most commonly to explain soil fungistasis a number of other suggestions exist, but the factors involved are not of widespread occurrence (Ko and Hora 1972a). Thus, while these may account for inhibition of spores in particular situations they are not as generally applicable as the theories of Dobbs and Hinson (1953) or Ko and Lockwood (1967).

Dobbs and Bywater (1957) suggested that volatile inhibitors might be present in some of their soils, but Lingappa and Lockwood (1961), using a wide range of soil types, failed to confirm this. However, Hora and Baker (1972b) demonstrated the production of a volatile inhibitor in alkaline soils. This inhibitor was

subsequently extracted (Hora and Baker 1972b) and shown to be heat stable (Romine and Baker 1972). Similarly, Kouyeas (1973) and Balis (1973) demonstrated a volatile inhibitor in soil which showed reduced activity after absorption in aqueous silver nitrate solution. A study of the ultra violet absorption spectrum (Balis 1973) suggested the material was similar to allyl alcohol which was shown to possess similar spore inhibiting properties.

It has been suggested that fungistasis might be due to staling products produced in ageing agar cultures (Park 1960, 1961), though this theory should be reconsidered in view of the more recent work on nutrient exhaustion in culture media (Ko and Lockwood 1967, Hsu and Lockwood 1969).

Lingappa and Lockwood (1960) extracted a fungitoxic fraction from soil containing lignin-like materials. Although these materials inhibited spore germination they were not implicated in the widespread soil fungistasis since this phenomenon may occur in soils low in organic matter (Lingappa and Lockwood 1962).

Recently spore germination inhibition in Hawaiian soil was demonstrated to be due to aluminium ions (Ko and Hora 1972a) but these were not implicated in general fungistasis.

The role of changes in redox-potential and pH in soil fungistasis has been examined (Jackson 1958, 1959; Lingappa and Lockwood 1961) but neither factor appeared to be important over normal physiological ranges encountered in soils. Some finely divided materials (bentonite, diatomaceous earths) prevented spore germination when assayed directly,

whilst clay and diatomaceous earth gave little or no inhibition when assayed indirectly. Thus, non-biological methods seem unlikely to explain general soil fungistasis.

No single theory has been accepted to explain soil fungistasis (Watson and Ford 1972) although the nutrient deficiency theory (Ko and Lockwood 1967) appears to have gained most general acceptance. A proportion of the work reported in the present study was concerned with germination responses of fungal spores in compost, this being an attempt to elucidate the mechanism of the specificity of this substrate for A. bisporus.

3.2 Germination response of fungal spores in compost

Germination of fungal spores in contact with compost was assessed as follows. Spores of test fungi were harvested from agar (3.5% w/v malt extract) cultures, suspended in sterile distilled water (4°C) and centrifuged three times at 3,000 r.p.m. for 10 minutes using an MSE Bench centrifuge to remove extraneous nutrients. Washed spores were finally suspended in sterile distilled water and applied to cellulose acetate membrane filters (Millipore, 0.45 µm pore size) under reduced pressure (Dobbs and Griffiths 1962). The concentration of spores was adjusted to achieve a separation between spores on membranes of 50-100 µm. Membranes were inverted on flattened surfaces of compost packed into 9.0 cm dia. glass Petri dishes. Compost and membranes were pressed firmly together to ensure good direct contact (Adams 1967) and the percentage germination of spores was assessed after

24 hours incubation at 25°C. Membranes were mounted in 0.05% w/v cotton blue in lactophenol and 300 spores counted.

Germination of spores of most fungi was inhibited when incubated in contact with compost (Table 1). The germ tubes which were produced showed signs of lysis within the 24 hour incubation period. Similar results were obtained when soil or EPS was used as the incubation substrate. However, sterilised substrates permitted high percentage germination of all spores tested. Thus, the behaviour of fungal spores in compost resembled the general pattern of behaviour of spores in soil (Dobbs and Hinson 1953; Lingappa and Lockwood 1964). However, Ko and Lockwood (1967) reported that some fungal spores, notably of genera not normally associated with soil, were capable of high percentage germination in soil. None of the fungi examined in this study belonged to this category.

Table 1

Germination response of fungal spores on sterile and non-sterile substrates

Test fungi	Substrates tested					
	Compost		E P S*		Soil**	
	natural	sterile	natural	sterile	natural	sterile
V.malthousei	0	100	2.3	99.3	0	98.3
D.dendroides	0	95.0	2.6	64.3	1.2	89.4
Penicillium sp.	4.6	92.6	5.3	89.3	2.3	84.3
Absidia sp.	0	98.6	0	73.0	0	96.2
C. tropica	5.6	82.0	0.6	88.6	1.4	86.3
Fusidium sp.	7.0	73.3	1.0	81.3	2.3	88.8
S.stemonitis	0	100	0	99.2	0.1	100
O.fimentarium	0	95.0	0	89.2	6.3	89.9
C.fimentarius	11.1	100	NT	NT	NT	NT
Ch.olivaceum	0	100	NT	NT	NT	NT
T.roseum	0	89.6	0.6	73.2	0.6	75.4

* E P S - expressly prepared substrate

** - obtained from pesticide free plot, Field Station, University of Bath

NT - not tested

4.0 Mechanism of inhibition of spore germination

4.1 Non-volatile germination inhibitors

a) Water soluble inhibitors: Extraction and assay procedures used previously by other workers to extract and demonstrate antibiotics from soil were used in an attempt to detect germination inhibitors in compost.

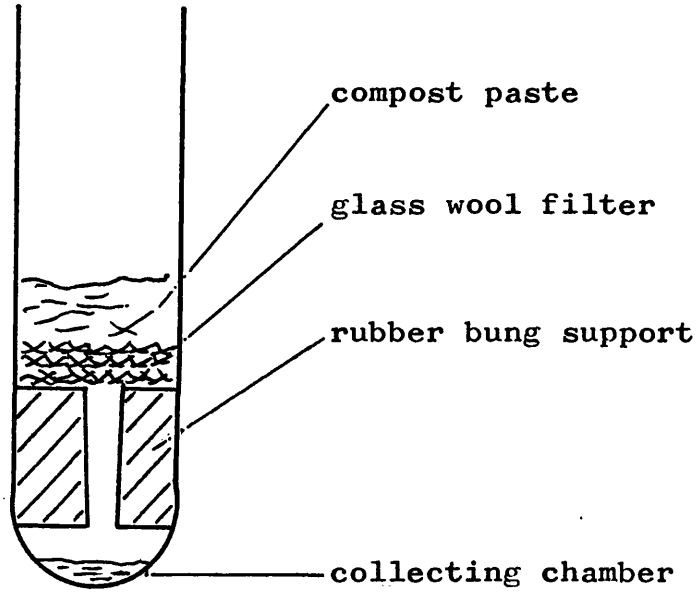
i. The centrifugation method (Grossbard 1952, Davies and Davies 1963) was modified for use with compost as follows. Compost was moistened and ground to a paste in a pestle and mortar with iced distilled water (10g. compost in 10 ml water). This was held at 4°C for 24 hr. to facilitate extraction by the water, packed into a centrifuge tube (Fig. 1), and centrifuged at 5,000 r.p.m. for 20 min. at 2°C using an MSE High Speed 18 centrifuge. Supernatants were filter sterilised (Millipore, 0.45μm pore size) and assayed for inhibitors using the whole plate method (Kruger 1961, Johnson and Curl 1972), and directly by adding spores to the extract. Nutrient agar, malt extract agar, compost agar and Brian and Hemmings agar (Brian and Hemming 1947) were used, the test fungi being Absidia sp., C. tropica, Fusidium sp., and S. stemonitis.

No inhibition of spore germination was noted in either the aqueous extract or in the agar assay technique.

ii. The cylinder plate method (Witkamp and Starkey 1956, Johnson and Curl 1972) was modified to test for water soluble inhibitors in compost.

Figure 1

Centrifuge tube for removing water from compost pastes



Polyethylene cylinders (1.5 cm. x 2.0 cm. internal diam.) were packed with a compost paste prepared as above. These compost cores were placed on sterile washed cellophane sheets (5 cm. x 5 cm.) over test agars previously seeded with test fungi.

Following incubation at 4°C for 24 hr. the compost and cellophane were removed and the agar plates incubated at 25°C. The same test media and fungi mentioned above were used.

Growth of test organisms was not inhibited on any plates confirming that water soluble inhibitors play no role in fungistasis in compost.

b) Water insoluble inhibitors: The importance of water insoluble inhibitors in compost was assessed using extraction procedures described by Lingappa and Lockwood (1961). Samples of compost (100g. fresh weight) were macerated in 500 ml extraction solvent using a domestic blender (Osteriser Co.) set at purée for 30 s. The homogenate was filtered through muslin and filter paper (Whatman No. 1) and the filtrate clarified by centrifugation at 5,000 r.p.m. for 5 min. Clear extracts were evaporated to dryness at 50°C under reduced pressure and stored at 1-4°C. The solvents used were methyl alcohol, ethyl alcohol, n-butanol, methyl alcohol:chloroform (1:1 v/v), diethyl ether, chloroform, water. All extractions were made at room temperature (16-24°C) and all extracts were redissolved in 1 ml extraction solvent prior to assay.

Three assay procedures were used; the filter paper disc method (Loo, Skell, Thornberry, Ehrlich, McGuire, Savage and Sylvester 1945) using 0.2 ml samples on 0.4 cm. dia. paper discs (Whatman Seed test paper); the hole-plate method (Kruger 1961) using 0.5 ml samples; the thin layer chromatographic plate technique. (Pet erson and Edgington 1969; Hough, pers. comm.) using 0.1 ml samples. After sample application all seeded test agars were held at 4°C for 12 hr. and then incubated at 25°C. The test media and fungi described above were used.

Extracts did not inhibit spore germination or growth of test organisms used. If inhibitors had been present in compost the range of solvents used would probably have extracted a proportion of one or more of these. Inhibitors have been extracted with solvents from sterile amended and non-amended soils inoculated with antibiotic producing micro-organisms (Lewis 1929, Grossbard 1948, 1952, Vasudeva, Singh and Iyengar 1962) from natural amended soil inoculated with such organisms (Wright 1952), or recovered from soil following prior addition of antibiotic (Gottlieb and Siminoff 1952)

The inability to demonstrate an inhibitor in compost in this study parallels the observations of Dobbs and Hinson (1953), Jackson (1958), and Lingappa and Lockwood (1961) in natural soil.

The capacity of organisms colonising peak heated compost to produce growth inhibitors was examined using in vitro methods. Thermophilic bacteria, actinomycetes,

and fungi were isolated from compost before and after peak heat as described previously. Bacteria and actinomycetes were isolated on compost agar*, Brian and Hemming agar, and nutrient agar, and fungi on compost agar, peptone dextrose agar and malt extract agar. The three layer technique of Herr (1959) was used to investigate antibiotic production *in vitro*. A basal layer of solidified water agar (0.5% w/v plain agar) in a Petri dish was covered with 8 ml molten (46-48°C) isolation agar to which was added 0.5 ml of an appropriate dilution of a compost suspension. These isolation plates were incubated at 45°C for 4 days. When required test organisms (*C. tropica*, *Absidia* sp., *Fusidium* sp, and *S. stemonitis*) were suspended in 10 ml molten agar (46-48°C) and poured into separate Petri dishes lined with sterile cellophane sheets. These were incubated at 25°C for 24 hr. Potato dextrose agar, malt extract agar and Brian and Hemming medium were used as test agars. When colonies of thermophilic micro-organisms were visible in the isolation agar the agar containing test fungi was transferred, with the aid of the cellophane sheets, onto this layer. After pressing these two layers together the whole plate was further incubated at 4°C for 12 hr. and then at 25°C for approximately 4 days. The dilution of compost suspension used in isolating thermophilic micro-organisms was chosen to provide about 30-40 colonies per plate.

* boil 1 kg fresh compost in 1 litre distilled water for 1 hr. Filter to clarify and add 12.0 g plain agar.

Only test plates of Fusidium sp. showed signs of growth inhibition (up to 0.5 cm. dia. inhibition zone). These results must be interpreted with caution in view of the observations of Hsu and Lockwood (1969) on nutrient exhaustion in agars.

In a further attempt to demonstrate antagonistic materials isolates of thermophilic bacteria, actinomycetes and fungi were screened in sterile compost with and without 0.5% w/w D-glucose as an energy source (Grossbard, 1948, 1952). After 2, 4 and 7 days incubation at elevated temperatures the compost was tested for diffusible inhibitors using the centrifugation and cylinder plate methods described above.

None of the thermophilic micro-organisms isolated from compost produced detectable amounts of spore germination inhibitors in vivo even when supplied with a supplementary energy source. However, Renoux-Blondeau (1959) has demonstrated antagonism between isolates of actinomycetes and other compost micro-organisms in phase-2 which he interpreted as playing a role in compost specificity.

The methods described above have been frequently used in attempts to detect soil antibiotics or inhibitors (Grossbard, 1948; 1952; Wright, 1952; Lingappa and Lockwood, 1961; Sneh and Henis, 1972). In an attempt to explain the inability to demonstrate inhibitors in natural soils, Baker (1968) suggested these materials might be adsorbed to clay or humus during extraction so

rendering their demonstration extremely difficult. Since compost is a substrate of high humus content (Grabbe, 1972), the efficiency of extraction procedures was examined using a model system. Spawn was prepared by inoculating moist sterile wheat grain with an isolate of Trichoderma viride known to produce an antibiotic (Supplied by T. Hunter, Long Ashton Research Station, University of Bristol). Peak heated compost was kept at 85°C in a humid atmosphere for 1 hr. before being cooled and inoculated with T. viride spawn. This heat treatment was necessary before growth of fungi, other than A. bisporus, occurred in compost. When fully colonised the compost was assayed, using Fusidium sp., and the thin layer chromatography plate technique described previously. Ethyl alcohol, methyl alcohol and water extracted an inhibitor from this compost (Table 2) suggesting that procedures used previously were not limiting factors in the detection of such metabolites.

Table 2

Extraction of germination inhibitors produced in compost by T. viride

Solvent	Diameter of Inhibition Zone(cm) of Fusidium sp. test media. (agars)				
	Nutrient	Compost	Brian & Hemming	Malt Extract	Potato dextrose
ethanol	0.5	1.0	1.0	2.0	2.0
methanol	0.5	1.0	1.0	2.0	2.0
water	0	1.0	0.5	1.0	0.5

4.2 Phenolic inhibitors of germination

From the results it appears that soluble and insoluble inhibitors are not responsible for compost specificity. However, Lingappa and Lockwood (1960, 1962) extracted lignin-like materials exhibiting fungitoxic properties from soil. The possibility that these types of materials occur in compost cannot be ignored, particularly in view of the suggestion by Grabbe (1968) that phenolic compounds and humic acids in compost may inhibit micro-organisms by interfering with oxidative phosphorylation.

The effect of lignin, lignin degradation products, and humic acid on the germination of test fungi was assessed using the method of Lingappa and Lockwood (1962). Native lignin was isolated from wheat straw using cold ethanol extraction (Brauns 1939), and humic acid by 0.5N NaOH extraction of peak heated compost (Grabbe 1972). Both were sterilised without heat (Sørensen 1962) and incorporated into test agars (plain agar buffered to pH6.4 with 0.1M phosphate buffer). Conidia of test fungi were streaked onto these agars and the percentage germination assessed after 24 hr. incubation.

High concentrations of vanillin, p-hydroxy-benzaldehyde, hydrocinnamic acid and verataldehyde inhibited spore germination (5×10^{-3}M) or caused a marked reduction in germ tube length (vanillic acid, chlorogenic acid, ferulic acid or syringaldehyde - Table 3). The germination response at lower concentrations (1×10^{-3}M) was less uniform.

Table 3

Effect of lignin, humic acid and lignin degradation products on fungal spore germination

Test compounds	Concentration (molar)	Test fungi					
		D.dendroides		C.tropica		T.roseum	
		%*	g.t.l**	%	g.t.l	%	g.t.l
hydrocinnamic acid	$1x10^{-3}$	100	400	100	1000	92	280
	$5x10^{-3}$	0	-	0	-	0	-
phenyl-2-propanone	$1x10^{-3}$	100	240	90	1500	40	50
p-hydroxy-benzaldehyde	$1x10^{-3}$	100	500	10	400	100	180
	$5x10^{-3}$	0	-	0	-	0	-
vanillin	$1x10^{-3}$	100	250	15	500	80	50
"	$5x10^{-3}$	0	-	0	-	0	-
vanillic acid	$1x10^{-3}$	100	220	20	400	100	100
"	$5x10^{-3}$	90	320	5	200	60	120
ferulic acid	$1x10^{-3}$	100	340	45	400	90	110
"	$5x10^{-3}$	95	60	7	500	16	30
Chlorogenic acid	$1x10^{-3}$	100	600	95	700	100	400
	$5x10^{-3}$	100	200	80	500	98	200
verataldehyde	$1x10^{-3}$	100	80	15	1500	10	60
"	$5x10^{-3}$	0	-	0	-	0	-
syringaldehyde	$1x10^{-3}$	100	340	36	300	20	30
"	$5x10^{-3}$	90	40	5	-	0	-
lignin	1.0 +	100	480	92	560	100	340
humic acid	1.0 +	100	550	95	640	100	350
control	-	100	600	80	500	100	400

* % = percentage spore germination
** g.t.l. = germ tube length (µm)
\+ used at 1.0 mg/ml

Germination of *D. dendroides* conidia was unaffected, though reduction in germ tube length occurred with certain compounds (Table 4). *Cephaliophthora tropica* and *T. roseum* germinated inconsistently on media containing phenolic compounds. Stimulation of germ tube growth of *C. tropica* occurred on media containing hydroxycinnamic acid, verataldehyde and phenyl-2-propanone. The effect of lignin and humic acids on spore germination was negligible but a slight reduction in extent of germ tube growth occurred.

These results indicate that high levels of free phenolic compounds (5×10^{-3}M), possibly derived from lignin degradation, inhibit germination of spores of a number of fungi and thus might contribute to compost specificity. The effects of these compounds on mycelial growth of *A. bisporus* and other saprophytes was then examined.

Basal medium containing 1.0% w/v D-glucose and 0.02% w/v L-asparagine was amended with phenolic compounds (1.5×10^{-4}M, 1.5×10^{-3}M), lignin or humic acid (1.0 mg/ml). Following multipoint inoculation of shallow liquid cultures dry weight of mycelium was assessed after 24 days incubation.

Growth of most fungi in liquid culture media containing p-hydroxybenzaldehyde and vanillin was reduced when compared with growth in phenol free media (Table 4). Lignin and humic acid had variable effects on mycelial growth at 1.0 mg/ml. and little can be deduced from the data. However, mycelium of *A. bisporus*

Table 4

Effect of phenolic compounds associated with lignin degradation, native lignin and humic acid on fungal growth in vitro.

Fungus	humic acid (1.0 mg/ml)	lignin (1.0 mg/ml)	Dry weight mushroom mycelium* vanillin** 1.5×10^{-4}	vanillin** 1.5×10^{-3}	vanillin** 3.0×10^{-2}	p-hydroxybenzaldehyde 1.5×10^{-4}	p-hydroxybenzaldehyde 1.5×10^{-3}	p-hydroxybenzaldehyde 3.0×10^{-2}
V.malthousei	104.2	98.4	83.0	67.7	0	59.9	80.7	0
T.roseum	98.4	112.4	76.4	28.9	0	90.7	45.1	0
S.stemonitis	109.6	109.4	88.1	70.3	0	128.1	93.4	0
Ch.olivaceum	100.3	106.2	84.7	43.1	0	80.4	86.8	17.8
S.thermophile	109.8	89.4	63.1	53.4	0	63.1	61.2	12.5
S.commune	97.4	92.4	79.1	116.0	16.9	68.5	130.6	63.0
P.versicolor	98.2	98.9	88.9	96.0	0	95.7	76.2	0
C.fimentarius	102.3	110.2	70.9	47.3	0	62.1	51.8	0
A.bisporus	110.2	104.3	168.9	104.2	0	226.4	132.9	0

* dry weight expressed as percentage of growth in phenol free media

** vanillin and p-hydroxybenzaldehyde, concentrations expressed as molarity

and S.commune grew better in media containing 1.5×10^{-4}M and 1.5×10^{-3}M vanillin and p-hydroxybenzaldehyde than in phenol free media. This would indicate that in substrates containing low concentrations of free phenolic compounds the mushroom might have a competitive advantage over other saprophytic fungi. During this study S.commune and P.versicolor were included for comparison since both produce laccase. If growth inhibition in phenol containing media was due to absence of laccase enzymes all three basidiomycetes should have remained uninhibited. Since P.versicolor was inhibited to a certain extent the significance of laccase in overcoming inhibition might be questioned. Grabbe (1968) suggested that the production of laccase by A.bisporus enables the mycelium to oxidise, neutralise and polymerise phenolic compounds,therefore the importance of this as a defence mechanism in compost should be reconsidered.

An attempt was made to detect phenolic materials in compost likely to inhibit the microflora and thus contribute to specificity. Compost was extracted according to Henderson (1955) and Whitehead (1964). All extracts were evaporated to dryness under reduced pressure, redissolved in a minimum volume of solvent and assayed for germination inhibitors using the thin layer chromatography plate technique. Spore germination occurred in

compost extracts obtained with single solvents, or a range of solvents, suggesting that compost does not contain sufficient easily extractable phenolic compounds to contribute to compost specificity. These findings are in agreement with those obtained earlier in this study. Thin layer chromatography was used in an attempt to determine the nature of phenolic components of compost extracts. Samples of compost extracted with chloroform were chromatographed using a two dimensional system with chloroform:acetic acid (5:1 v/v) as solvent 1, and ethyl acetate:benzene (45:55 v/v) as solvent 2. Phenolic materials were located using u.v. light or diazotised sulphanilic acid (Stahl 1969). Pure samples of phenolic compounds (B.D.H. Ltd.) likely to result from lignin degradation were chromatographed for reference purposes (Appendix 3). Nineteen distinct spots (Fig.2) were separated but only caffeic acid and syringaldehyde could be identified. Caffeic acid is involved in the synthesis of lignin (Goodman, Kiralay and Zaitlin,1967) and is a product of lignin degradation, in common with syringaldehyde (Lingappa and Lockwood 1962; Henderson, 1963). The level at which these compounds were extracted from compost was obviously not sufficient to inhibit spore germination.

The stimulation of mycelial growth of A.bisporus by vanillin and p.hydroxybenzaldehyde is paralled by Marasmius foetida growing on gallic acid (Lindberg and Korjus 1949). While the ability of fungi to utilise phenolic compounds as carbon sources has been known for some time (Henderson and Farmer, 1955) the growth promoting

Figure 2

Two dimensional chromatogram of chloroform extracted phenolic materials from peak heated compost.

Solvent 1 10% v/v acetic acid in chloroform

Solvent 2 45% v/v ethyl acetate in benzene

Spots located with U.V. light or diazotised sulphanilic acid.

effects of such materials are seldom reported.

Thus phenolic compounds appear to be unimportant in compost specificity. Substances which inhibit spore germination have been extracted from high-volatile bituminous coals (Mills 1959) and from peat in smaller quantities (Rogoff and Wender 1961) but materials of this kind are not present in mushroom compost.

4.3 Volatile inhibitors

Recently the suggestion that volatile inhibitors may be responsible, at least in part, for soil fungistasis (Dobbs and Bywater 1957) has received support (Hora and Baker 1972a.b.; Balis 1973; Kouyeas 1973). The production of volatile metabolites by micro-organisms which have a stimulative or depressive effect on the activity of other micro-organisms has been studied by a number of workers (Hutchinson, 1971, 1973; Fries 1973).

Some time ago Eger (1962) suggested that A.bisporus may produce volatile metabolites which inhibit the growth of compost inhabiting saprophytic micro-organisms but no information is available regarding the production of volatile metabolites by micro-organisms in peak heated compost. This possibility was examined using the method of Eger (1962).

Compost was packed into 9.0 cm dia. glass Petri dishes (30g. fresh weight/dish) and pressed flat. One millilitre suspensions of compost were spread evenly over the surface of freshly poured plates of nutrient

or malt extract agar. When dry the plates were inverted over the compost. One set of three dishes was completely sealed around the edges with masking tape (3M brand No. 1222) and another partially sealed with two 2.0 cm lengths of tape to allow aeration to occur. Sterile compost was used in controls in place of peak heated compost.

After incubation for three days the mean numbers of colonies developing on the inverted agar was assessed and related to dry weight of compost used in preparing the suspensions. Any volatile materials released by peak heated compost had no effects on the numbers of developing fungi, bacteria or actinomycetes. (Table 5).

Table 5

Effect of volatile metabolites from peak heated compost on numbers of compost micro-organisms*

Test organisms	Plates incubated over		
Mean Numbers.g.dry wt.$^{-1}$	Peak heated compost	Sterile compost	Standard error of mean (p=.05)
bacteria	43.0×10^8	46.5×10^8	12.7
actinomycetes	22.9×10^7	21.2×10^7	7.4
fungi	18.4×10^4	24.9×10^4	7.8

* results for unsealed plates; results for sealed plates included in Appendix 2.

Since volatile or non-volatile inhibitors are unlikely to account for the inhibition of spore germination of fungi in compost the phenomenon was investigated further as follows.

4.4 Nutritional status of spores in relation to compost specificity

The importance of nutritional requirements of spores in compost in relation to spore behaviour was examined in view of the findings of Ko and Lockwood (1967). Spores of test fungi were removed from culture agar, washed three times in distilled water to remove free nutrients (Ko and Lockwood 1967) and applied to membrane filters as previously described. Membranes were floated onto glass distilled water and incubated for 18 hr. before percentage germination was assessed.

Two types of germination response were noted (Table 6). Four species of fungi (D.dendroides; C.tropica; Fusidium sp., and T.roseum), showed high percentage germination, while the remainder showed little or no response. The possibility that germination might have been influenced by nutrient deficiency was examined. Membrane filters containing spores of the same test fungi were floated on sterile 0.5% w/v nutrient solution and incubated for 18 hr.

The majority of fungal spores, except those of Ch.olivaceum and C.fimentarius, germinated when supplied with a single carbon or nitrogen source, or both (Table 6). Three groups of spores could be distinguished on their germination behaviour. One group (V.malthousei, Penicillium sp., S.stemonitis, O.fimentarium) required simple nutrients for germination while spores of other fungi (D.dendroides, C.tropica, Fusidium sp., T.roseum)

Table 6

Relation between fungal spore germination and nutrient availability*

Fungi	Percentage spore germination							
	H_2O	$(NH_4)_2SO4$	asparagine	urea	glucose	sucrose	starch	glucose + asparagine
V.malthousei	3.3	96.3	90.6	75.0	93.0	11.0	93.0	NT
D.dendroides	84.6	99.0	98.3	91.3	97.0	3.6	97.6	NT
Penicillium sp.	1.3	0	0	4.3	0	0	0	61.0
C.tropica	89.2	2.8	93.6	99.0	93.6	0.3	84.6	NT
Fusidium sp.	100	81.3	93.6	93.0	71.0	16.6	91.6	NT
S.stemonitis	5.3	16.6	1.0	5.1	13.5	0	9.3	98.0
T.roseum	97.3	88.0	94.6	98.3	95.0	0.3	87.3	NT
O.fimentarium	0.6	0	0	89.0	0	0	0	NT
C.fimentarius	0	0	0	0	0	0	0	0
Ch.olivaceum	0	0	0	0	0	0	0	0

*Nutrient solution 0.5% w/v

NT - not tested

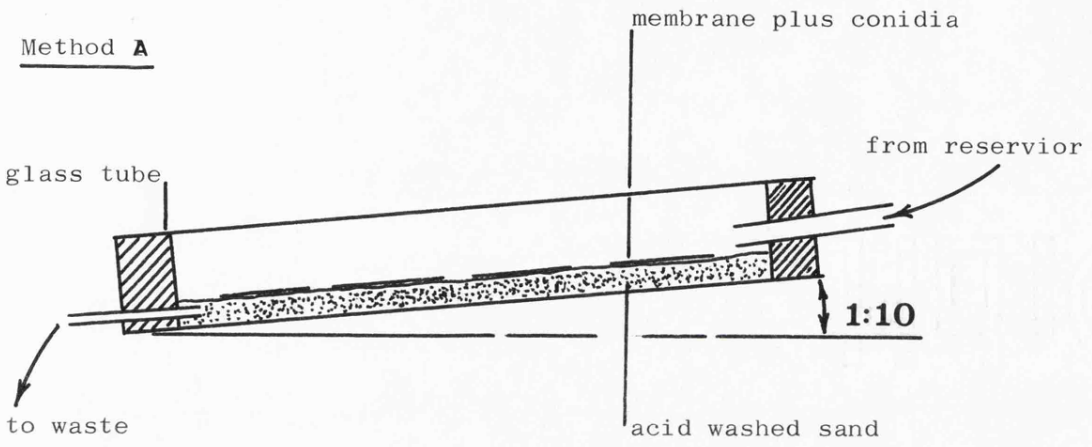
were nutritionally independent. A third group (Ch.olivaceum, C.fimentarius) failed to germinate, possibly indicating a requirement for more complex nutrients, or the presence of endogenous germination inhibitors. The nutritionally dependent and independent spores resembled those observed by Ko and Lockwood (1967) and were examined further.

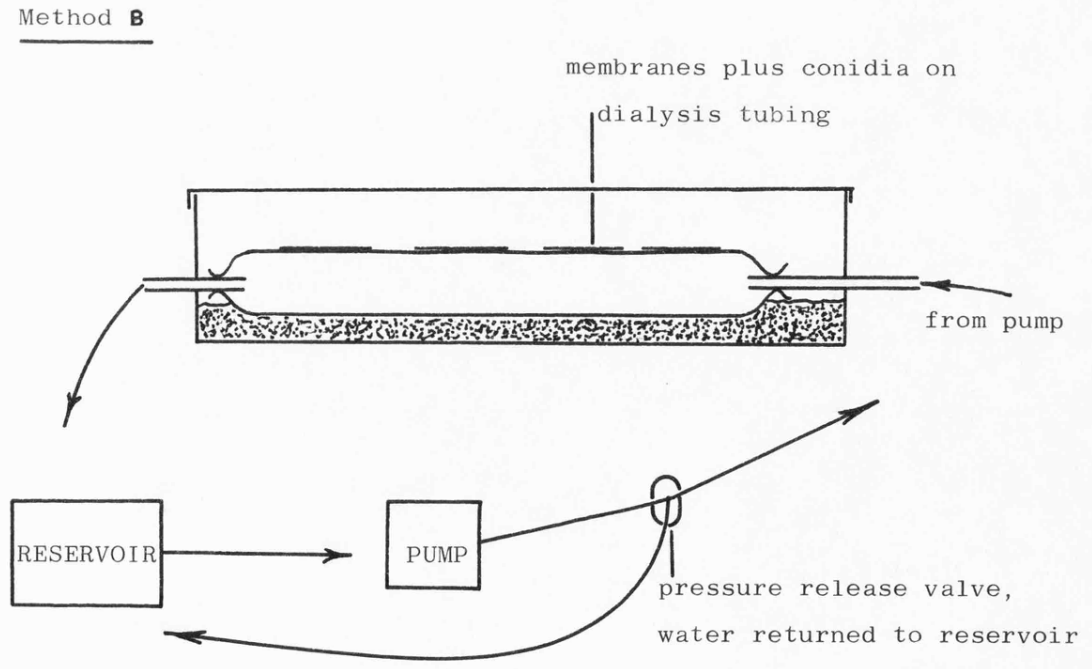
Probably for some fungi non-germination of spores in compost can be related to the need for simple nutrients as suggested by Ko and Lockwood (1967). However, in some cases no correlation of this kind can be made (Table 6). The suggestion that microbially induced leaching of metabolites essential for germination (Ko and Lockwood 1967) may account for inhibition in these fungi i.e. C.tropica, Fusidium sp., D.dendroides, and T.roseum was examined.

The effect of physical leaching on germination of spores was assessed using two model systems. For leaching at a low rate, system A (Fig.3) was used. A glass tube (30cm x 1.5 cm int.dia.) was half filled with acid washed sand (British Drug Houses Ltd.) and autoclaved after sealing both ends with rubber bungs containing a central glass tube (0.5 cm dia.). Filter membranes containing spores of test fungi, prepared as described previously, were placed on the sand (the tube being held at a gradient of 1 in 10). This was irrigated with sterile distilled water, care being

Figure 3

LEACHING APPARATUS





taken to regulate the flow rate to avoid washing spores from the membrane. After leaching with water at 24± 2°C for 18 hr. the percentage germination of conidia was assessed. The flow rate was expressed as the rate of flow through $1cm^3$ of sand, per unit of time. Because of the limit imposed on the rate of leaching by this system the apparatus of Lloyd and Lockwood (1966) was used in another experiment. This technique (Fig. 3B) has been used to investigate inhibition of germination of spores (Lloyd and Lockwood, 1966), sclerotia (Hsu and Lockwood 1973), and to examine materials removed from these fungal propagules by leaching (Bristow and Lockwood 1973; Jackson and Knight 1975; Sneh and Lockwood, 1976).

Inhibition of spore germination occurred in both leaching systems (Table 7). However, only the large conidia of C.tropica were sensitive to the low rate of leaching ($1.4cm^3/cm^3/hr$). Germination of conidia of D.dendroides and Fusidium sp. was inhibited only at the higher rate of leaching ($35.0cm^3/cm^3/hr$).

Table 7

Percent germination of conidia exposed to leaching

Test fungi	Control*	Method A	Method B	Germination rate (hr)**	Spore size (μm)
C.tropica	93.3	0.3	NT	8	17.8x38.3
D.dendroides	100	96.4	4.2	18	9.6x28.8
Fusidium sp.	97.0	97.6	1.6	18	2.4x 4.8
leaching rate ($cm^3/cm^3/hr$		1.4	35.0	-	-

* Method A without water flow
** Time for 90% germination
NT Not tested

These observations appear to contradict the finding of Steiner and Lockwood (1969) who suggested that nutritionally independent large spores with a rapid rate of germination are less sensitive to fungistasis than small spores with slower rates of germination. They suggested that large spores might have a larger internal nutrient supply and therefore be less affected by removal of smaller amounts of nutrient by leaching.

These contradictory observations might be reconcilable since Steiner and Lockwood's (1969) data depended on microbial leaching whilst data in this study were obtained by mechanical removal of nutrients from spores. Whilst an incubation period would be required to allow microbial colonisation of the spore in soil before effective leaching of nutrients occurred this would not be required in a mechanical system. The ease of removal of nutrients, and the rate at which it occurs in relation to the rate of germination would be important in inhibiting germination. Also the high rate of water flow used in method B need not reflect the effective leaching rate since Ko and Lockwood (1970) indicated that this was an inefficient method of removing metabolites. Evidence to support the idea that leaching may occur in compost was the next aspect examined.

Glucose agar discs (0.1% w/v), 0.5 cm dia., were sandwiched between two sterile membrane filters

(Millipore) drained of excess moisture. These were placed on compost, plain agar, sterile sand or sterile activated charcoal (B.D.H. Ltd) and pressed to ensure good contact. Conidia of the three test fungi were similarly sandwiched between sterile membrane filters and added to the same substrates. After incubation for 18 hr. the amount of glucose remaining in the agar discs was determined using the Nelson (1944) and Somogyi (1952) assay, and the percentage germination of spores assessed.

Materials producing a large reduction in germination also caused a large percentage loss of glucose from agar discs (Table 8). Conversely, a low level of glucose removal was associated with a small percentage reduction in germination. Activated carbon acted in a way similar to compost, both in removal of glucose from agar and inhibition in spore germination.

The role of micro-organisms in maintaining negative nutrient gradients was examined using the techniques of Lingappa and Lockwood (1961). Glucose agar discs were incubated between sterile membrane filters as described above, but microbial activity at the compost-membrane-agar interfaces was inhibited by including 0.1 mg/ml of the microbial inhibitors $HgCl_2$, cycloheximide, Penicillin G, or streptomycin sulphate in the glucose agar, and by incubating non-sterile compost at $4^{o}C$. Sterile compost was used as a control.

Table 8

Relation between leaching and germination of nutrient independent spores

Organism	Percentage germination							
	plain agar		sand		carbon		compost	
	%	(μm)	%	(μm)	%	(μm)	%	(μm)
C.tropica	94	650	100	1000	0	-	0	-
D.dendroides	100	600	100	550	0	-	0	-
Fusidium sp.	100	170	100	180	0	-	0	-
glucose removed (%)		24.0		39.0		92.0		76.0

% = percentage spore germination ; μm = germ tube length

Table 9

Relation between leaching and microbial activity

Substrate	Treatment	Percentage glucose removed
peak heated compost	none	74.9
"	autoclaved	8.6
"	held at 4°C	8.2
"	$HgCl_2$	9.9
"	Penicillin G.	42.5
"	streptomycin S04	33.5
"	cycloheximide	35.1

Microbial inhibitors used at 1.0 mg/ml in glucose agar.

Restriction of microbial activity at the compost-membrane-agar interface resulted in a large percentage reduction of glucose removed from agar (Table 9). Agar discs held on compost at 4°C or on sterile compost lost equivalent amounts of glucose, suggesting that some loss by diffusion can occur. The results of Penicillin G and streptomycin sulphate treatment indicate that bacteria are not entirely responsible for leaching. Fungi appeared to be actively involved in removing glucose if most bacteria and actinomycetes were inhibited (Table 9).

The results indicate that compost possesses the potential to remove nutrients from added substrates more rapidly than passive diffusion. On adding spores to soil Lingappa and Lockwood (1964) observed an increase both in soil respiration and microbial numbers. Thornton (1953) postulated the existence of a "hyphosphere" where micro-organisms use mycelial exudates but Lockwood (1964) was among the first to suggest that microbial population increases were linked with the release of nutrients from fungal spores in soil.

The effect of adding nutrient independent spores to compost was examined using the method of Lingappa and Lockwood (1964). Warburg flasks (15 ml) were packed loosely with 4.0g fresh weight compost. Spores of test fungi were removed from culture media using a suction gun (Fig.4) modified from a pressure spray gun (Shandon Co.). By regulating suction pressure a good separation between mycelium and spores was achieved,

Figure 4

Modified Shandon spray gun for
removing dry spores from agar cultures

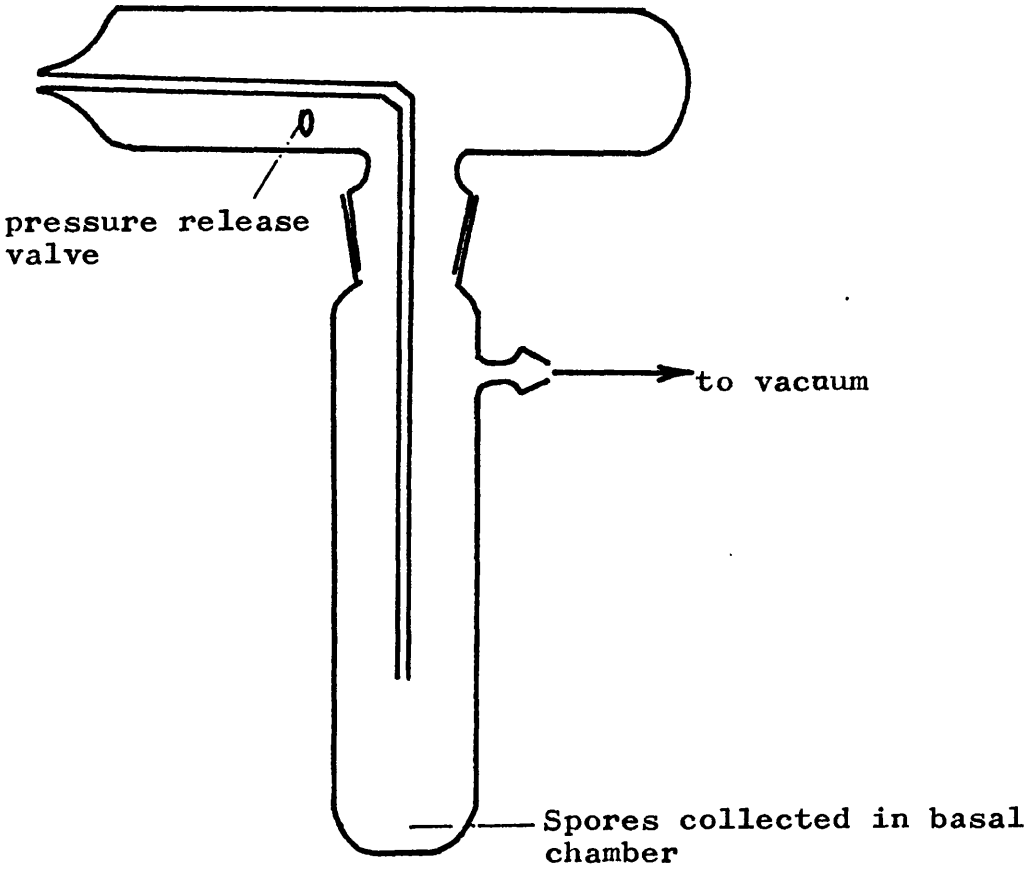


Figure 5

Effect of adding fungal conidia and nutrients to compost - changes in respiration.

■ C.tropica

□ Fusidium sp.

▲ glucose

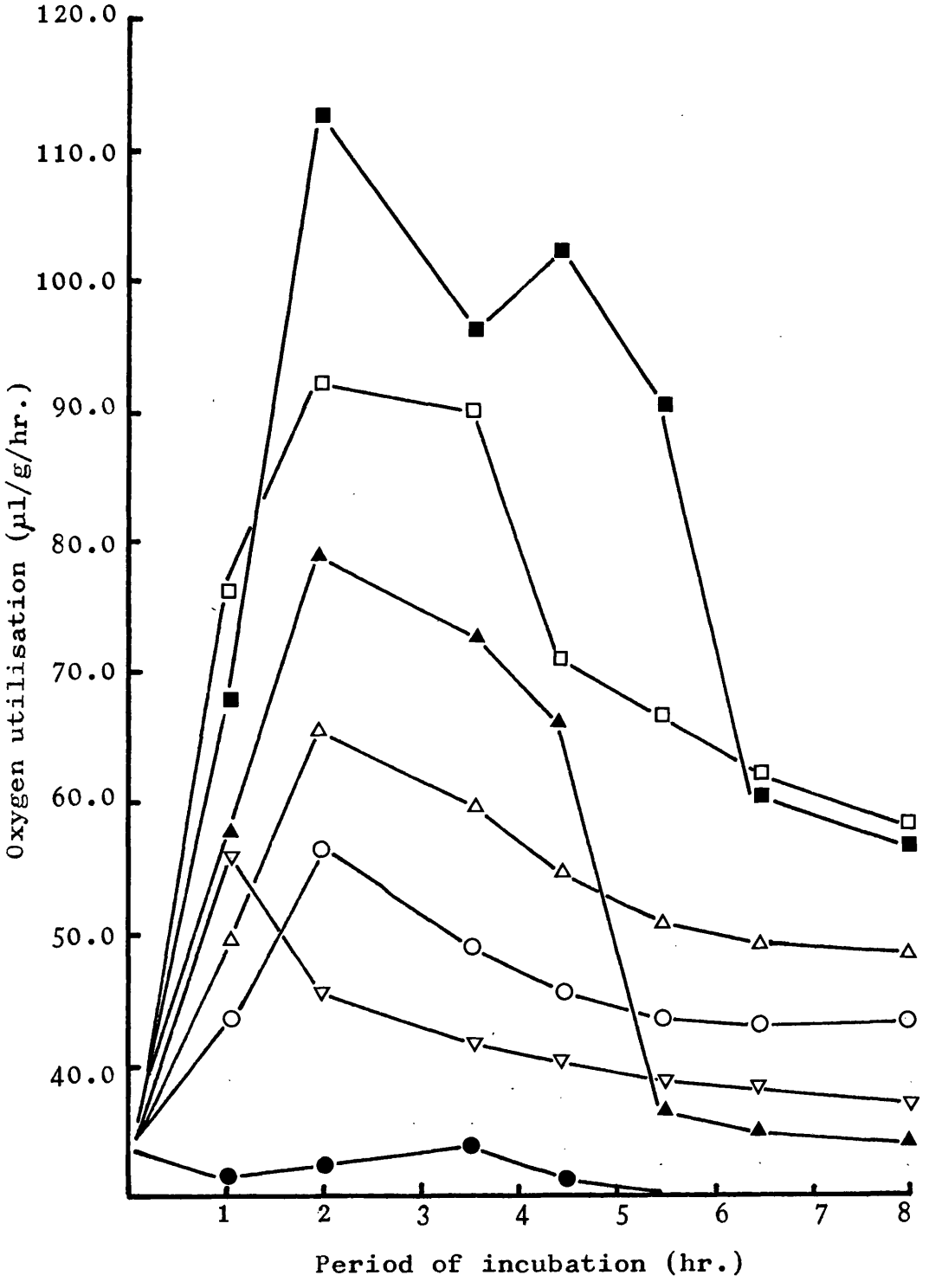
△ Penicillium sp.

○ S.stemonitis

▽ water

● control

Figure 5


120.0
110.0
100.0
90.0
80.0
70.0
60.0
50.0
40.0
Oxygen utilisation (µl/g/hr.)
1
2
3
4
5
6
7
8
Period of incubation (hr.)

the spores being collected dry in the basal chamber. These spores were hand mixed with compost in the Warburg flasks (100 mg. spores/flask). Flasks were equilibrated in the Warburg water bath for 30 min. and readings taken at 1 hr. intervals during incubation at 21°C. Nutrient dependent spores of Penicillium sp. and S.stemonitis (100 mg/flask), glucose and peptone (10 mg/flask), and water (0.01 ml/flask) were also used for comparison. Incubation was continued until a steady state of respiration was achieved in most flasks (8 hr.).

The addition of spores, nutrients and water to compost resulted in increased respiration within the compost (Fig.5). However, the extent of the increases over the control varied. The initial increase in respiration rates in compost amended with Penicillium sp., S.stemonitis, glucose and peptone was rapidly lost. The increase in respiration rate in compost amended with the nutrient independent spores of C.tropica and Fusidium sp. was greater, both in extent and duration, than in any other amended compost. Since few of the added spores germinated in compost (Table 1) it must be assumed that the increase in respiration in spore amended compost was due either to a high rate of endogenous respiration in spores, or to increased compost microbial activity resulting from release of nutrients by fungal spores. Increases in respiration in composts amended with C.tropica and Fusidium sp., and their sensitivity to mechanical leaching (Table 7) appeared to be similar to the behaviour of nutrient independent spores in soil

(Lingappa and Lockwood 1964; Ko and Lockwood 1967).

The numbers of bacteria and actinomycetes in compost was examined after the 8 hr. incubation period noted above. Samples of compost (1.0g fresh weight) were taken from each flask and the number of bacteria and actinomycetes estimated using the soil dilution plate method described previously. No marked changes in numbers of bacteria could be detected in amended composts (Table 10). The numbers of actinomycetes counted were low and variable and have been omitted.

Table 10

Effect of adding spores and nutrients on numbers of compost bacteria

Compost treatment	Mean number bacteria ($x.10^9$)*/g.dry wt.
glucose	4.2
peptone	3.9
water	3.5
Penicillium sp.	2.8
S.stemonitis	5.8
Fusidium sp.	4.2
C.tropica	8.3
Control	5.2

* Mean of six replicates LSD (5%) = NS

These findings are in contrast to those of Lingappa and Lockwood (1964) who recorded a three to tenfold increase in total microbial numbers from spore amended soils. However, having been subject to peak heating most mesophilic micro-organisms in compost would

have been killed but mesophilic heat resistant spores and endospores would survive this treatment. It is suggested that nutrients from added spores may allow these heat resistant propagules to germinate, thus accounting for the increased respiration recorded, but would not be sufficient to allow extensive cell division.

To satisfy the nutrient deficiency theory the substrate must be very low in free nutrients to maintain a negative nutrient gradient from nutritionally independent spores (Ko and Lockwood 1967). The nutrient status of compost was therefore examined by suspending 1.0g samples in 50 ml sterile distilled water ($4^{o}C$) and shaking on a wrist action shaker (Griffin Ltd.) set at No. 3 for 10 min. The resulting suspension was filtered and reducing sugars (Nelson 1944; Somogyi 1952), total carbohydrate (Morris 1948) and α-amino acids (Lee and Takahashi 1966) determined. Autoclaved compost, soil, and natural soil were similarly analysed for comparison.

Compost is not low in extractable free nutrients (Table 11) but these figures need to be interpreted with caution since water extraction of materials with such a high organic matter content probably results in removal of nutrients not normally available to saprophytic micro-organisms, e.g. intracellular metabolites.

Table 11

Nutrient status of peak heated compost and soil

Substrate	Compost		Soil	
	sterile	non-sterile	sterile	non-sterile
Carbohydrate*	28.9	16.7	1.2	0.1
reducing sugar*	1.6	1.9	0.09	0.01
amino acids**	15.1	7.4	0.86	0.12

* mg/g glucose equivalent
** µg/g glycine equivalents

The low levels of nutrients in soil, and the increase in these caused by sterilisation have been noted previously (Ko and Lockwood 1967). Since it proved impossible to determine the nutrient status of compost by extraction another approach was attempted.

If the nutrient deficiency theory does explain inhibition of microbial activity in compost then addition of free nutrients should result in increased microbial activity as occurs in soil (Dobbs and Hinson 1953; Ko and Lockwood 1967). If low microbial activity contributes towards the successful growth of A.bisporus in compost then addition of nutrients should increase this and reduce the extent of growth of the mushroom. This was examined as follows.

Dry nutrients (60.0, 12.0, or 1.2 mg/g) were added to compost (40g fresh weight) which was then packed into growth tubes (30 cm x 3.5 cm internal dia.). One end was plugged with cotton wool (Robinson and Sons Ltd.,

Chesterfield), the other end inoculated with 5 grains A.bisporus spawn (D.621 strain), covered with 2.0 cm compost to prevent desiccation of spawn, and plugged with cotton wool. All tubes were incubated horizontally and the extent of mycelial colonisation measured after 7 and 14 days. Extent of mycelial growth into compost was taken as an indication of acceptability of the compost to A.bisporus. A manometric method (Lingappa and Lockwood 1964) with 0.5g (dry weight) compost per flask was used to measure rate of respiration within compost, with and without amendment, after 24 hr. and 72 hr. incubation. Quantitative estimates of microbial numbers in these composts were made using the dilution plate method.

Addition of carbon and nitrogen sources to compost resulted in a reduction in mycelial colonisation by A.bisporus. Glucose (60.0, 12.0 mg/g compost) and sucrose (60.0, 12.0 and 1.2 mg/g compost) caused significant reductions ($p = 0.05$) in mycelial colonisation (Table 12). This reduction was still evident after 14 days growth. A similar effect was noted for starch amendments but this was not detected after 14 days. There was no correlation between compost respiration rate, after 24 hr. or 72 hr., and acceptability of the compost to growth of A.bisporus.

Addition of nitrogen containing compounds to compost caused a more noticeable effect, both on respiration in compost and on mycelial growth (Table 13). Ammonium sulphate at all rates of addition resulted in significant reductions ($p=0.05$) in mycelial growth of A.bisporus in

Table 12

Relation between composts amended with carbon sources and extent of mycelial colonisation by A.bisporus

Amendment	Conc. (mg/g)	Oxygen utilised (µl/g/hr)		mycelial growth (cm)	
		24 hr.	72 hr.	7 days	14 days
glucose	60.0	76.4	67.3	0	1.3
	12.0	64.2	67.5	0.6	6.4
	1.2	89.3	65.8	1.3	8.2
sucrose	60.0	73.2	74.4	0.2	4.3
	12.0	79.4	69.8	1.3	6.5
	1.2	81.3	65.8	2.4	5.4
starch	60.0	NT	47.4	0.8	7.8
	12.0	NT	41.9	1.2	7.8
	1.2	NT	46.3	2.2	7.7
Control	0	54.5	59.4	2.7	8.1
LSD. (p=.05)	-	24.2	NS	0.9	0.9

NT = not tested

Table 13

Relation between composts amended with nitrogen sources and extent of mycelial colonisation by A.bisporus

Amendment	Conc. (mg/g)	Oxygen utilised (µl/g/hr)		mycelial growth (cm)	
		24 hr.	72 hr.	7 days	14 days
$(NH_4)_2SO_4$	60.0	NH_3 ↗	NH_3 ↗	0	0
	12.0	34.2	18.32	0	Chaet
	1.2	23.7	62.7	1.8	8.1
asparagine	60.0	73.2	132.4	0	0
	12.0	48.5	86.2	0	Chaet
	1.2	56.9	65.2	0.8	6.3
chitin	60.0	NT	40.9	1.5	7.4
	12.0	NT	35.1	1.7	7.6
	1.2	NT	48.3	2.4	8.1
Control	0	54.5	59.4	2.7	8.1
LSD (p=.05)	-	19.8	21.3	0.8	0.9

NT : not tested
↗ : indicates evolution of free ammonia
Chaet: compost fully colonised by Ch.olivaceum

compost after 7 days. The two highest rates of addition (60.0 and 12.0 mg/g) resulted in the evolution of free ammonia. Asparagine similarly caused a significant reduction ($p = 0.05$) in mycelial growth in compost after 7 and 14 days. Rates of respiration in composts were also increased following such additions, a significant rise ($p = 0.05$) being noted after 72 hr. incubation in substrates amended with 60.0 and 12.0 mg/g asparagine. Chitin (60.0 and 12.0 mg/g) resulted in reduced mycelial colonisation of compost by A.bisporus but this effect was not noticed after 14 days growth. Both ammonium sulphate and asparagine when added to compost at 12.0 mg/g resulted in complete inhibition in the growth of A.bisporus the compost being completely colonised by Ch.olivaceum.

With high levels of nitrogen containing amendments reduced growth of A.bisporus occurred in compost. This reduction may have resulted from the evolution of ammonia in compost, this being toxic to A.bisporus (Stoller 1945, Yod er and Sinden 1953), or from increased microbial activity possibly with subsequent increased leaching of hyphae. Microbial leaching of hyphae in soil is thought to cause autolysis in certain fungi (Ko and Lockwood 1970). Inhibition of mycelial growth in compost amended with carbon containing substances probably results from increased microbial activity and consequent hyphal leaching. To examine this aspect of the inhibition of

Table 14

Quantitative changes in compost microflora caused by nutrient additions

Amendment	Conc. (mg/g)	Nos. of bacteria ($\times 10^6$/g dry wt)	Nos. of fungi ($\times 10^4$/g dry wt)
asparagine	60.0	420.0	2.0
"	1.0	110.0	<1.0
$(NH_4)_2SO_4$	60.0	179.0	25.3
"	1.0	50.5	1.0
chitin	60.0	390.0	8.3
"	1.0	31.7	< 1.0
glucose	60.0	150.5	15.2
"	1.0	21.4	2.4
sucrose	60.0	198.0	26.3
"	1.0	31.2	2.8
starch	60.0	182.4	25.2
"	1.0	42.3	1.8
Control	0	8.3	< 1.0
LSD (p=.05)		4.2	NA

NA - not analysed because of insufficient data

mycelial growth further the numbers of organisms in non-amended and amended compost were assessed after 3 days incubation using the dilution plate method.

Adding carbon and nitrogen sources to compost caused increases in bacterial and fungal populations (Table 14) suggesting that easily assimilated nutrients are absent from compost. This is in agreement with the results obtained from remoistened air dried soil (Rovira 1953, Stevenson 1956), and with those from soil amended with nutrients (Rovira 1956, Katznelson and Raualt 1957). This upsurge of microbial activity upon adding soluble easily assimilated materials is a requisite of the nutrient deficiency theory (Ko and Lockwood 1967).

5.0 The Growth of *A.bisporus* in compost

Having suggested reasons why most fungi fail to germinate and grow in compost it is necessary to explain the ability of *A.bisporus* to grow freely in this medium.

The requisite for a certain inoculum potential for substrate colonisation was first demonstrated in the growth of *Coprinus sterquilinus* on dung balls (Buller 1931). Similarly Blair (1943) observed that *Corticium (Rhizoctonia) solani* would grow saprophytically through soil if the inoculum potential was adequate. When considering the function of mycelial strands of *Psalliota hortensis* in substrate colonisation, Garrett (1953) noted that a certain inoculum potential was required for successful colonisation of soil. If

inoculum levels were minimal colonisation of substrates was delayed.

In the present study inoculum potential is used to describe the energy of growth (Garrett 1953) of the fungus at the mycelium substrate interface.

Successful growth of A.bisporus in compost may be a function of the quantity of spawn used. Grain spawn, in addition to providing a large number of inoculation points also provides a source of nutrient which could substantially affect the rate of colonisation of compost. The effect of grain on mycelial colonisation was considered.

5.1 Inoculum potential and compost specificity

Spawn was prepared from A.bisporus and other fungi as follows. Wheat grain, supplemented with 0.2% w/w L-asparagine and 0.5% w/w $CaCO_3$, was autoclaved for 30 min. on two consecutive days and inoculated with test fungi. When the grain was fully colonised with mycelium it was used as spawn.

Glass growth tubes (30cm x 4.0 cm int.dia.) were packed with 40g (fresh weight) compost. The bottoms were plugged with cotton wool (Robinson and Sons, Chesterfield). Varying amounts of spawn (0.1, 1.0 and 3.0g) were added to the tops, covered with 2.0 cm compost and plugged with cotton wool. All tubes were incubated horizontally and the rate of mycelial growth through compost measured. An indicator line was marked on the growth tube to denote the region of spawn and

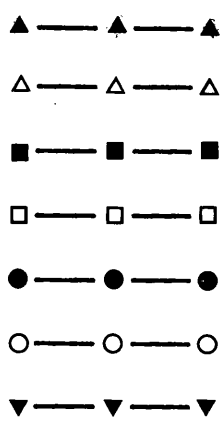
any growth of mycelium into the compost from this region recorded during incubation at 25°C. Ten predetermined points along the mycelial front around the periphery of each tube were measured and the mean taken.

The extent of mycelial growth in compost from the highest inoculum (3.0g) is shown in Fig. 6. Here growth of A.bisporus was vigorous and accompanied by a change in colour of compost from black to light brown. Although Coprinus fimentarius was able to grow in compost to about the same extent both the vigour, i.e. number and thickness of hyphal strands, and growth curves differed. Advance of the mycelial front of C.fimentarius was accompanied by lysis of the older parts of the mycelium. Growth of Chaetomium olivaceum into compost was weak and accompanied by perithecial production, possibly indicating a deficiency of suitable nutrients. Mycelial extension into the compost by Trichothecium roseum and Trichoderma viride was restricted to less than 5.0 cm, the nutrients for this limited growth probably deriving from the cereal grain rather than compost. No growth into compost was recorded from spawn of other fungi at this inoculum level.

Reduction in levels of inoculum (Fig. 7) resulted in reduced mycelial extension of both C.fimentarius and Ch.olivaceum but not A.bisporus. Although the vigour of growth of A.bisporus was reduced at 0.1g the amount of inoculum had little effect on the rate of colonisation of compost. After 15 days growth the mycelium of

Figure 6

Rate of mycelial colonisation of peak heated compost from 3g.grain spawn.



▲—▲—▲ A.bisporus

△—△—△ C.fimentarius

■—■—■ Ch.olivaceum

□—□—□ T.roseum

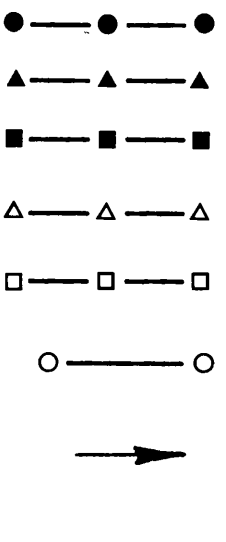
●—●—● Trichoderma sp.

○—○—○ S.stemonitis, P.versicolor

▼—▼—▼ S.commune, Fusidium sp.

Figure 7

Rate of mycelial colonisation of peak heated compost from 0.1 and 1.0 g. grain spawn.



●—●—● A.bisporus 3.0g.

▲—▲—▲ " 1.0g.

■—■—■ " 0.1g.

△—△—△ C.fimentarius 1.0g.

□—□—□ Ch.olivaceum 1.0g.

○———○ Common point for all other fungi tested

——→ indicates extent of mycelial growth from spawn before growth in compost

Figure 6

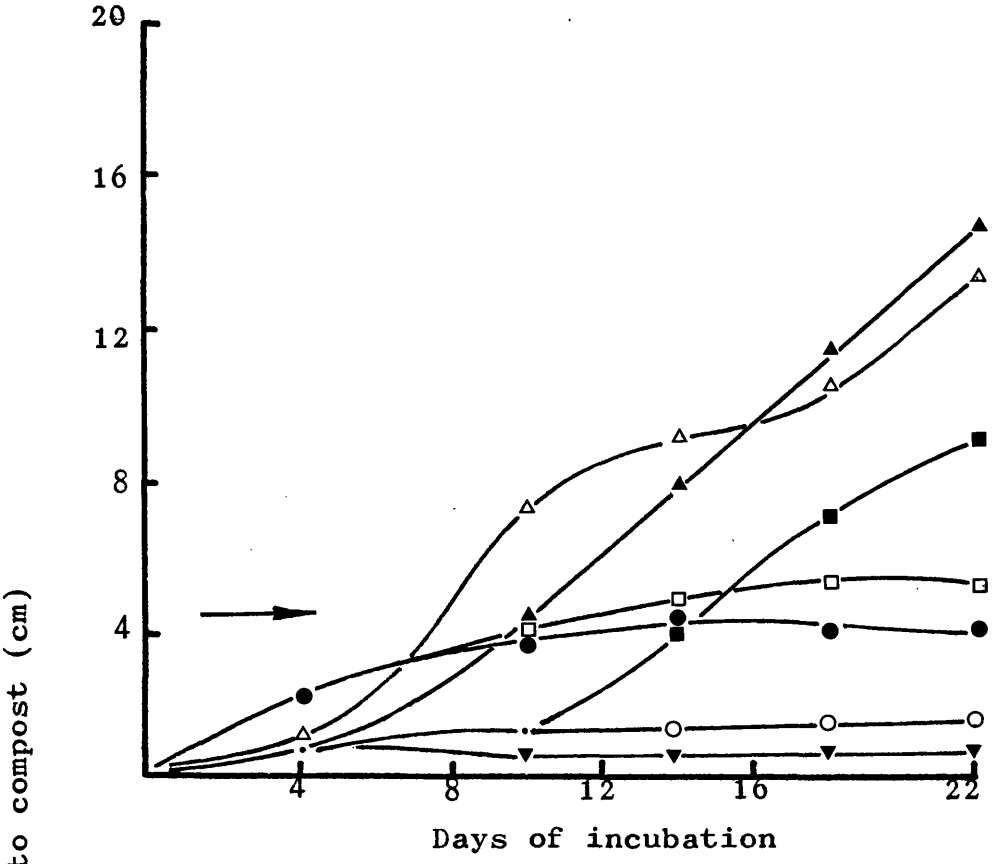
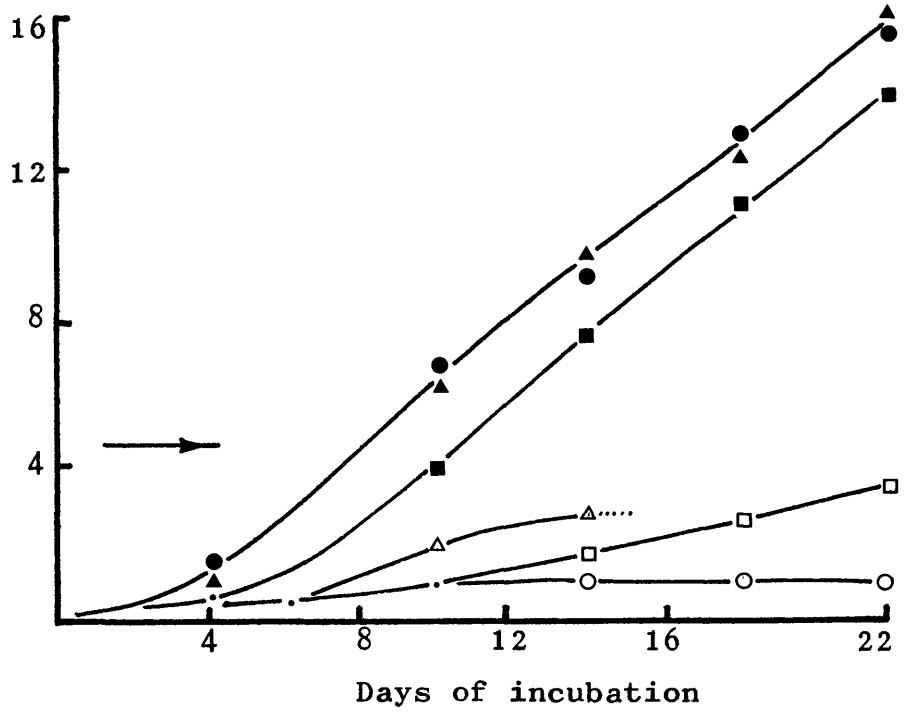


Figure 7



C.fimentarius had lysed, probably reflecting a severe deficiency of nutrients.

These results suggest that inoculum potential is not an important factor in the ability of A.bisporus to grow in compost, or the inability of most other fungi to grow in the substrate, except at unrealistic inoculum levels. Such high levels are unlikely to be encountered in commercial practice because of the lethally high temperatures involved in compost preparation (Lambert and Ayers 1957). To investigate these ideas mycelium and spores of the fungi used in the present study were exposed to elevated temperatures and the degree of survival measured.

Fresh peak heated compost was sterilised as previously described, packed into 250 ml beakers (50g/beaker) and inoculated with mycelium of test fungi. These were covered with aluminium foil and incubated at 25°C. Four beakers were inoculated with each test fungus. After mycelial colonisation of compost two beakers were removed, one was incubated further at 45°C, another at 55°C. When sporulation had occurred the two remaining beakers at 25°C were similarly incubated at 45°C and 55°C. After incubation at elevated temperatures for 4 days 10 pieces of compost (1 cm. long) were removed from each beaker, plated onto malt extract agar and incubated at 25°C to detect any growth from mycelium or spores.

Exposing compost to temperatures of 55°C for 4 days apparently eliminated all test fungi except Sporotrichum thermophile (Table 15). This isolate was

Table 15

Thermal sensitivity of mycelium and spores of test fungi in compost

Fungus	Growth form: (spores-S, mycelium-M)	Isolation on agar after 4 days at 45°C	55°C
Ch.olivaceum	M	X	-
	S	X	-
C.fimentarius	M	X	-
"	S	X	-
Penicillium sp.	M and S	/	X
T.roseum	M	X	-
"	S	X	-
S.stemonitis	M	X	-
"	S	/	X
V.malthousei	M	X	-
"	S	X	-
C.tropica	M	X	-
"	S	X	-
Absidia sp.	M	X	-
"	S	X	-
D.dendroides	M	X	-
"	S	X	-
S.thermophile	M	/	/
"	S	/	/
S.commune	M	X	-
P.versicolor	M	X	-
Fusidium sp.	M S	X	-

X = no isolation

/ = positive isolation

- = not tested

observed growing in commercial compost immediately after peak heat and would be expected to survive these test conditions. The mycelium and spores of all other test fungi were equally sensitive to incubation at 55°C. However, at 45°C the spores of Penicillium sp., S.stemonitis and Fusidium sp. were able to survive for 4 days. The other fungi which may occur in compost on mushroom farms, i.e. C.fimentarius and Ch.olivaceum, did not appear to survive incubation for 4 days at 45°C or 55°C. However, the lack of isolation of viable spores or mycelium from these fungi after incubation at elevated temperatures cannot be taken as proof of heat sensitivity. Basidiospores of C.fimentarius and ascospores of Ch.olivaceum are extremely difficult to germinate on culture agars, hence the lack of recovery may reflect a lack of spore germination through the absence of suitable essential nutrients or environmental stimuli. Since few fungi seemed able to survive the elevated temperatures of peak heat, except possibly C.fimentarius, Ch.olivaceum and Sporotrichum thermophile, high inoculum levels of weed moulds are not likely to occur in compost following this treatment.

5.2 Competitive Saprophytic Ability of A.bisporus

Garrett (1956) suggested that the results of competitive struggles between soil micro-organisms for colonisation of a particular substrate will not only depend on the inoculum potential of the micro-organisms

concerned but also on their competitive saprophytic ability. This ability of a fungus is "the summation of physiological characteristics that make for success in saprophytic colonisation of dead organic substrates" (Garrett 1956). He considered that four factors were likely to affect competitive saprophytic ability *viz.* rate of spore germination and mycelial growth, production of antimicrobial factors, tolerance to these types of materials produced by other competing micro-organisms, and competition for nutrients. Another factor to be considered is the susceptibility of fungal mycelium to fungistasis imposed by microbially induced leaching (Ko and Lockwood 1970). However, the degree of competitive saprophytic ability shown by fungi can vary widely from one type of substrate to another and is therefore substrate specific (Dwivedi and Garrett, 1968). Thus studies of the saprophytic ability of *A.bisporus* in compost were made in the hope that a knowledge of the factors contributing to this may help elucidate the mechanisms of compost specificity. This approach was handicapped by the lack of detailed knowledge of the chemical composition of peak heated compost. Previous work has suggested that cellulose (Waksman and Nissen 1932), hemicellulose (Treschow 1944), 'ligno-protein complex' (Waksman and Iyer 1932), 'nitrogen-rich lignin-humus complex' (Gerrits *et al* 1965) humic acids (Grabbe 1972), extracellular bacterial polysaccharides (Stanek 1972) and microbial cell residues (Gerrits et al 1965; Hayes 1968) might contribute to the nutrients for mushroom growth in compost.

5.3 Changes in wheat straw during composting and cropping

Changes in the structure of wheat straw resulting from composting and the growth of A.bisporus were studied in an attempt to determine the nature of the major nutritional substrates involved. Initially it was decided to study these changes using histological techniques.

Samples of straw were removed from freshly prepared piles, two day old stacks, compost at the end of phases 1 and 2, at the end of spawn run (21 days), and after the second flush of sporophores. Pieces of straw were cut into 1-2 cm lengths, freeze dried and coated with ethyl cellulose, applied as a 10% w/v solution in chloroform. Coating straw in this way successfully retained surface layers that would normally have become dislodged during embedding. When dry the straw segments were embedded in paraffin wax (Jensen 1962) and 10-20 µm thick sections cut using a rotary microtome (Swift Co.).

The histological changes occurring in straw during compost preparation and utilisation are shown in figures 8-14. Surface and internal materials were absent from straw in freshly prepared piles (Fig.8); the cellulose containing phloem cell walls were detectable, though some degradation had occurred. After two days in stacks (phase-1) a slight surface covering of unidentified material was visible on straw and all phloem cell walls had disappeared (Fig.9). At the end of phase-1 a distinct layer of dark material had accumulated on the surface of straw (Fig.10). After peak heat a noticeable increase in

Figure 8: Section of wheat straw from fresh piles four days before stacking. Note: No surface or internal colonisation by micro-organisms; some phloem cell walls are visible. Crystal violet-safranin stain (x100)

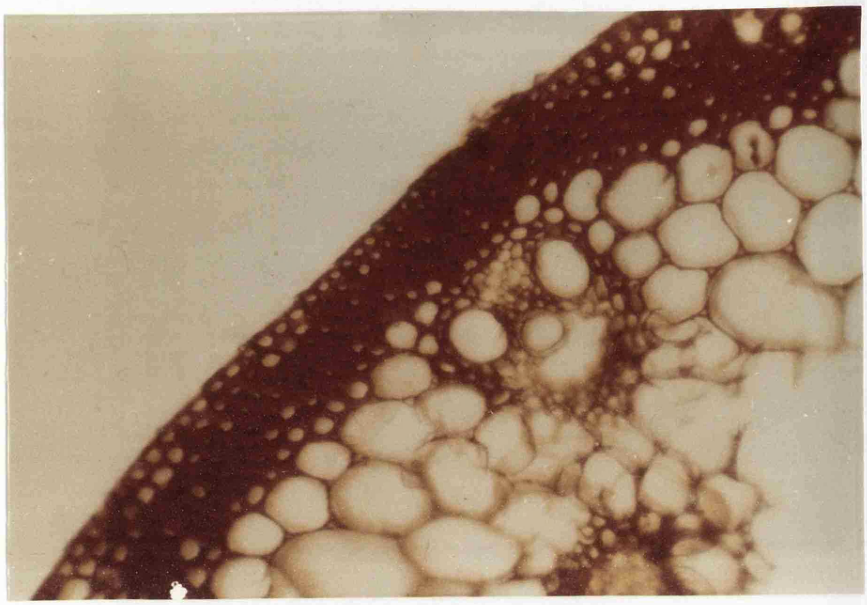


Figure 9: Section of wheat straw from compost after 2 days in stacks. Note: Slight surface covering ; phloem cell walls degraded. Crystal violet-safranin stain (x100)

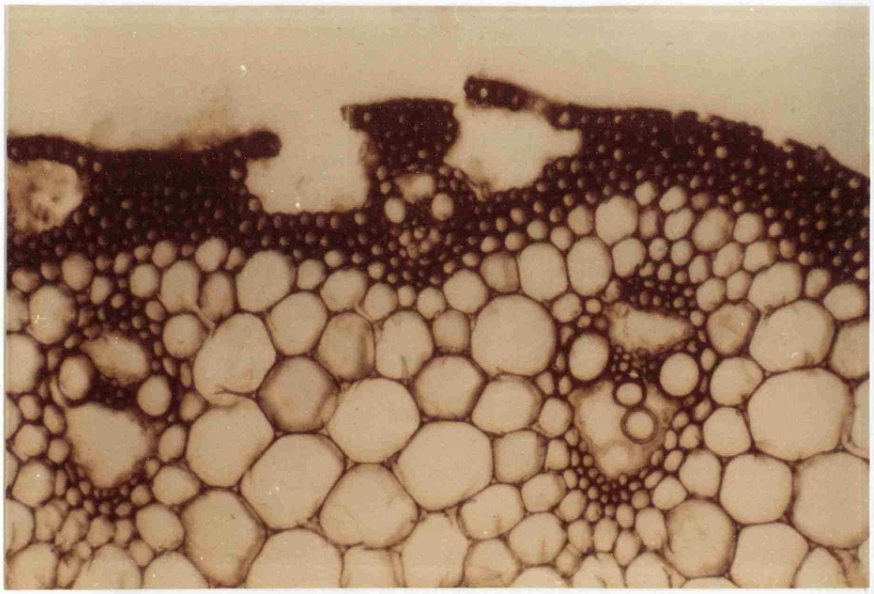


Figure 10: Section of wheat straw from compost at the end of phase-1. Note: thick layer of surface colonisation. Cotton blue/ lactophenol (x100)

Figure 11: Section of wheat straw from compost after phase-2, peak heat. Surface and internal colonisation very noticeable, especially the fungal mycelium (presumably thermophilic). Aniline blue (x100)

Figure 12: Section of wheat straw at the end of phase-2. Note the thick surface layer still present. Understained with cotton blue/lactophenol (x100)

Figure 13: Section of wheat straw after spawn run (day 14). Surface layers removed but some internal colonisation still visible. Bismark brown (x100)

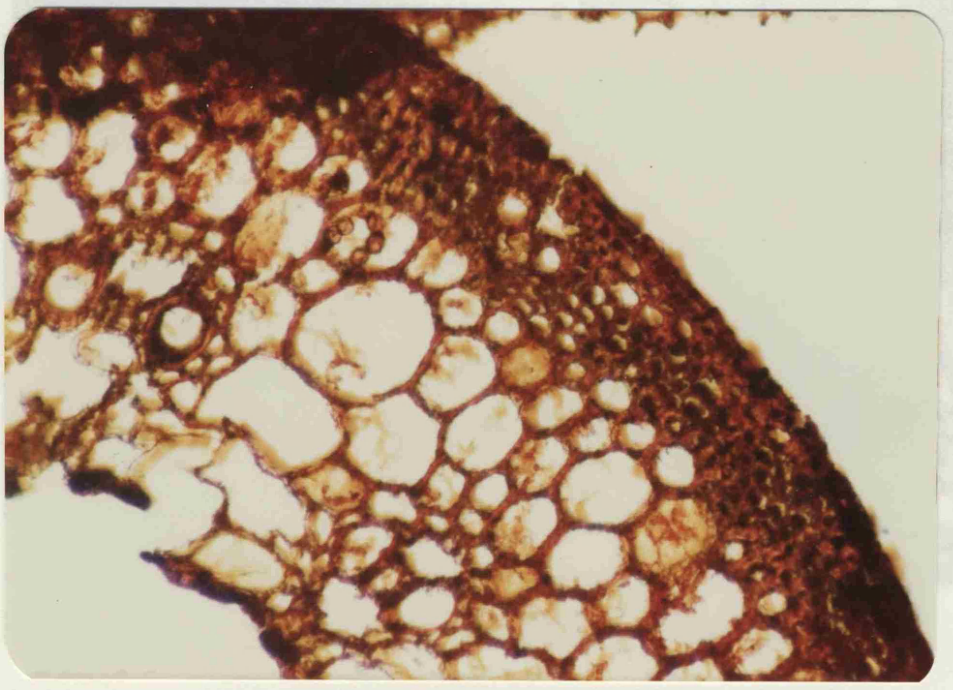
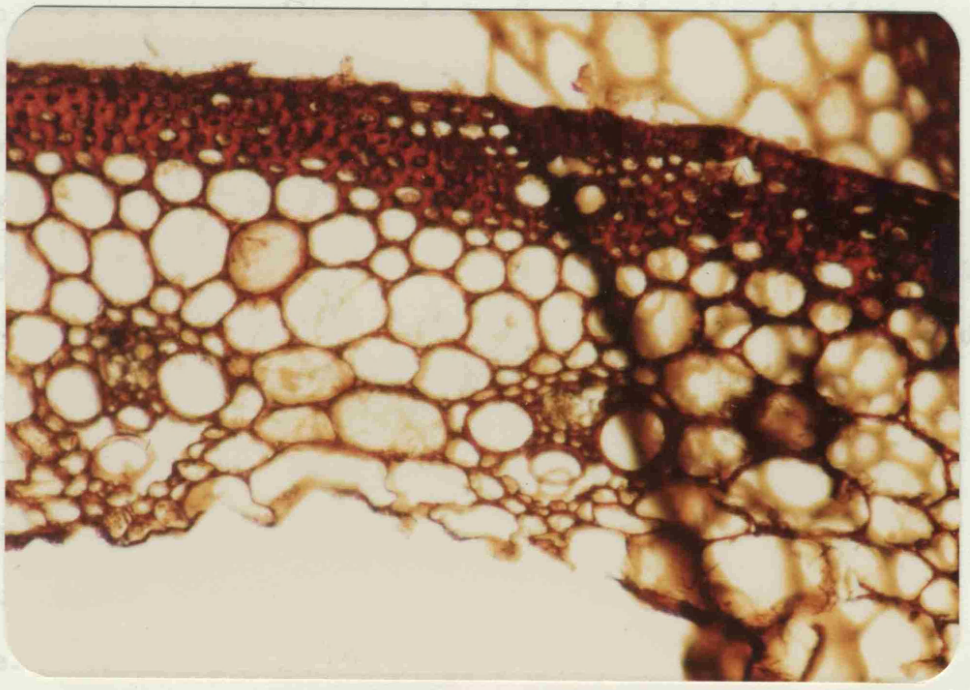


Figure 14: Section of wheat straw after cropping (flush 2). Surface and internal colonisation much reduced. Structure of straw appeared relatively unchanged. Bismark brown (x100)



internal microbial colonisation, presumably of thermophilic micro-organisms, was evident (Fig.11). An understained section from straw at this stage revealed that the surface layers on the straw were still present after peak heating (Fig.12). During spawn running i.e. the period of rapid colonisation of compost by mycelium, these surface layers were removed (Fig.13) and the amount of thermophilic mycelium present inside the straw vessels appeared to decrease. The section from straw after cropping (Fig.14) indicated that mass structural degradation of straw did not occur, the stained brown-red areas suggesting that a considerable amount of lignin remained. However, these techniques would not detect any changes which might have occurred in the lignin molecules or the closely associated cellulose and hemicellulose.

The changes brought about in surface layers of peak heated compost was studied using a Cambridge Stereoscan S4 transmission electron microscope. Samples of straw were freeze dried and covered with Ag/Pt before being used.

The surface of fresh straw was relatively free from colonisation by micro-organisms. Distinct ridges and lenticels in the cuticle could be identified (Fig.15). After peak heating a thick coating of microbial material was visible on the straw surface (Fig.16) in which it was possible to recognise spores and mycelium, presumably thermophilic fungi (Fig.17). The areas marked with arrows (Fig.17) may represent

Figure 15

Surface of uncomposted straw. The cuticle appears ridged with lenticels clearly visible. Relatively little surface debris is visible. x 290.

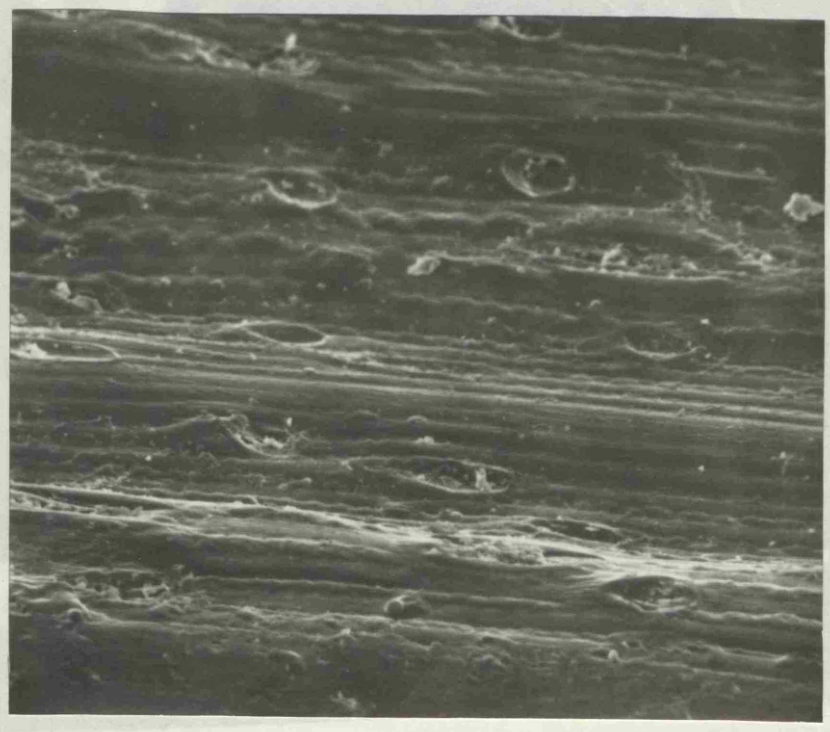


Figure 16

Surface of unspawned composted straw (after phases-1 and 2). Large amount of surface colonisation evident. x60 (See Figure 17).

Figure 17

Higher magnification (x300) of Figure 16, centre. Straw surface covered with microbial residues, noticeably fungal mycelium and spores and possibly bacterial surface cover (arrowed).

Figure 18

Surface of spawn run and cropped (flush 3) compost; relatively free of microbial remains, and lenticels again visible. Note strands and aggregations of mushroom mycelium (x70).

straw surface colonisation by bacteria. As a result of spawn run and mycelial growth in compost this surface layer was removed (Fig.18) and the surface of the straw was again exposed and appeared relatively unaltered since lenticels and ridges were again visible on the surface.

These observations confirm that straw in mushroom compost is exposed to considerable surface and internal colonisation by thermophilic micro-organisms during composting. Through spawn run and cropping this microbial mass decreases substantially. No decrease occurred in unspawned compost. Contrary to expectations little gross cytological change appeared to have occurred in the overall structure of the straw during the growth of A.bisporus as seen with the light microscope. However, biochemical changes in the composition of cell walls might well have occurred. Phloem cell walls were rapidly degraded during stacking and phase-1 but a great deal of lignified tissue remained even after cropping. These results prompted an investigation into the nature of the surface layers which developed during composting, and the part these played in the nutrition of A.bisporus.

The black surface layer was scraped from composted straw and examined microscopically. Smear preparations revealed that the bulk of this material was micro-biological in nature. Fragments of fungal mycelium, and spores of Torula sp. and Humicola sp. were common. A large proportion of the smear preparations consisted of a mucilaginous material surrounding bacterial cells.

The use of alcian blue-basic fuchsin (Novelli 1953) resulted in this material staining as extracellular bacterial polysaccharide. Small fragments of actinomycete mycelium were also noted in these smears. Since this layer disappeared during spawn running it is possible that the mushroom mycelium may utilise microbial cells and/or extracellular microbial slime as a nutrient source. If such cells are utilised a reduction in numbers of micro-organisms isolated from compost might be expected. A rapid reduction in thermophilic organisms during cropping was reported by Fordyce (1970) and a decrease in numbers of actinomycetes was later noted by Lacey (1973). It is possible that these decreases resulted from natural senescence, utilisation of micro-organisms by A.bisporus as a nutrient source, or to the production of toxic materials by the mushroom. This work was repeated using different isolation media and sampling techniques, and including unspawned compost as a control.

Spawned and unspawned composts were treated as described for sporophore production. Samples of compost were taken at weekly intervals by removing 5g quantities (fresh weight) from four well spaced areas within the growth containers. Numbers of mesophilic and thermophilic micro-organisms were estimated by the dilution plate method.

The population of mesophilic micro-organisms remained relatively stable throughout the period of study in both spawned and unspawned compost (Fig.19)

Figure 19

Variation in numbers of mesophilic micro-organisms in spawned and unspawned compost.

Figure 20

Variation in numbers of thermophilic micro-organisms in spawned and unspawned compost.

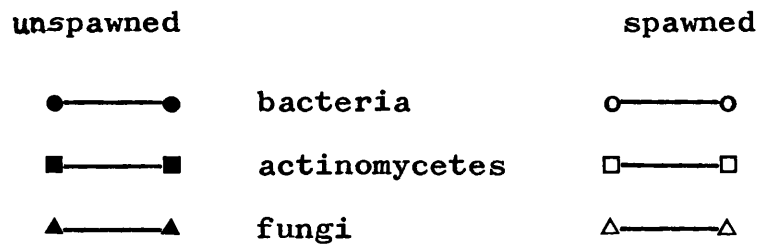


Figure 19 Mesophiles

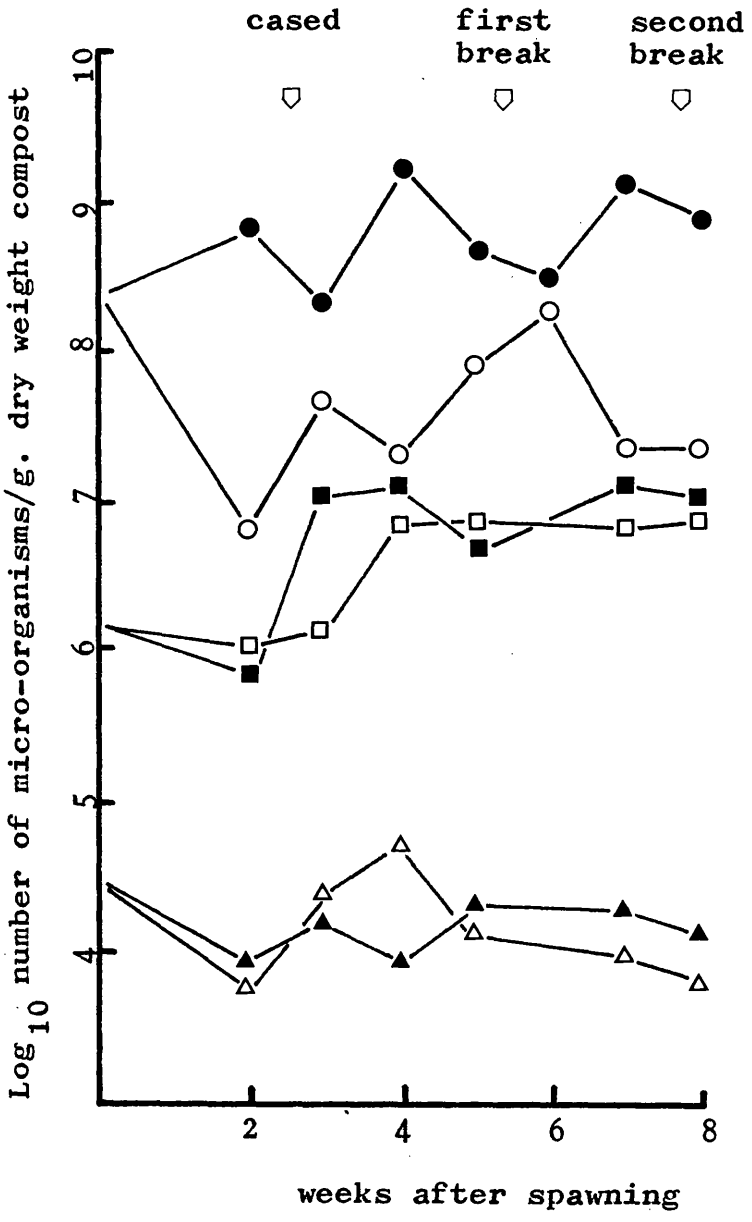
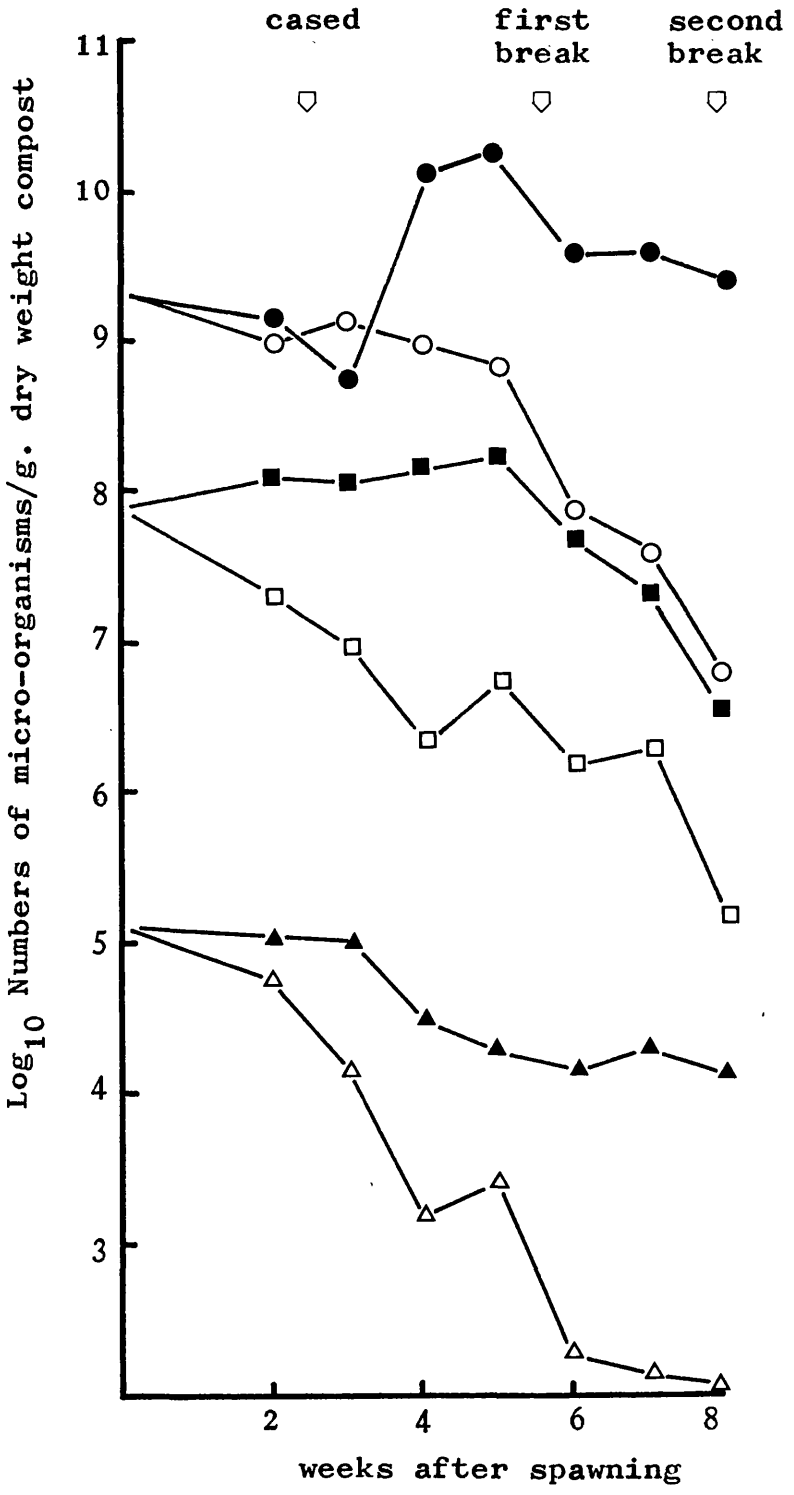


Figure 20 Thermophiles



but the numbers of mesophilic bacteria were consistently lower in spawned compost. Although no detailed assessment was made of qualitative changes in mesophilic bacteria differences in the morphology of bacterial colonies were detected on isolation plates by the end of the experiment.

The estimates for numbers of thermophilic bacteria, actinomycetes and fungi in unspawned compost were variable throughout the study but a gradual decrease with time was noticed. (Fig.20). A more pronounced decrease in these numbers occurred in spawned compost. The number of fungal propagules isolated appeared to decrease more rapidly during the period of mushroom initial production and subsequently. Actinomycete numbers showed a steady decline during mushroom growth, while the numbers of bacteria decreased most rapidly after the first flush. Thus the trend of population changes would indicate that spawned compost was subject to decreases in thermophilic bacteria, actinomycetes and fungi directly linked with the presence of mushroom mycelium. These decreases may have been caused by natural or induced death of the cells or degradation by A.bisporus. It is interesting to note that the decrease in numbers of thermophilic micro-organisms parallels the decrease in thickness of the surface layers on straw in compost suggesting that the mushroom could have actively degraded microbial cells in this fraction of compost. To investigate this possibility the degradation of thermophilic cells in vitro was examined.

5.4 Degradation of thermophilic micro-organisms by A.bisporus

Isolates of thermophilic fungi, bacteria and actinomycetes were cultured separately in 8 litre conical flasks. Bacteria were grown on mineral salts medium (Wilkinson, Dudman and Aspinall 1955) supplemented with 0.2% w/v L-asparagine; actinomycetes on mineral salts medium (Lilley and Bull 1974); and fungi in malt extract broth (3.5% w/v) with mycological peptone (0.5% w/v). All cultures were aerated and agitated by sterile filtered air at 100 ml/min.

Cells were harvested by sterile centrifugation, using an MSE High Speed 18 centrifuge, and washed three times in sterile distilled water before use. Fungal mycelium was macerated in a domestic blender (Osteriser Co.) set at purée for 30s. before being washed as above. Washed cells were added to basal medium with 1.2% w/v plain agar. Fungi (5.0 mg/ml), bacteria (4.0 mg/ml) and actinomycetes (4.0 mg/ml) were either added to agar before autoclaving or to molten agar at 80°C. The thermophilic cell containing media were dispensed into 9.0 cm dia. glass Petri dishes (20 ml/dish) and inoculated with A.bisporus mycelium or other test fungi previously cultured on potato dextrose agar. Any clearing in the opaque media was recorded after incubation at 25°C.

Agaricus bisporus grew better on autoclaved than non-autoclaved substrates (Table 16). Coprinus fimentarius degraded autoclaved bacterial cells (Fig.21)

Table 16

Growth of A.bisporus and other test fungi on thermophilic micro-organisms supplied as major nutrient sources

(+ indicates clearing in agar, - indicates no clearing)

Test fungi	autoclaved cells in agar			non-autoclaved cells in agar		
	bacteria	actinomycetes	fungi	bacteria	actinomycetes	fungi
A.bisporus	+	+	+	+ (slow)	-	++
C.fimentarius	++	+	-	-	-	-
Ch.olivaceum	+	-	-	-	-	-

Other fungi examined included T.roseum, D.dendroides, Oedocephalum sp., S.thermophile, Trichoderma sp. and a Fusidium sp. All failed to produce clearing in test agars.

Figure 21

Degradation of autoclaved thermophilic bacterial cells by Coprinus fimentarius

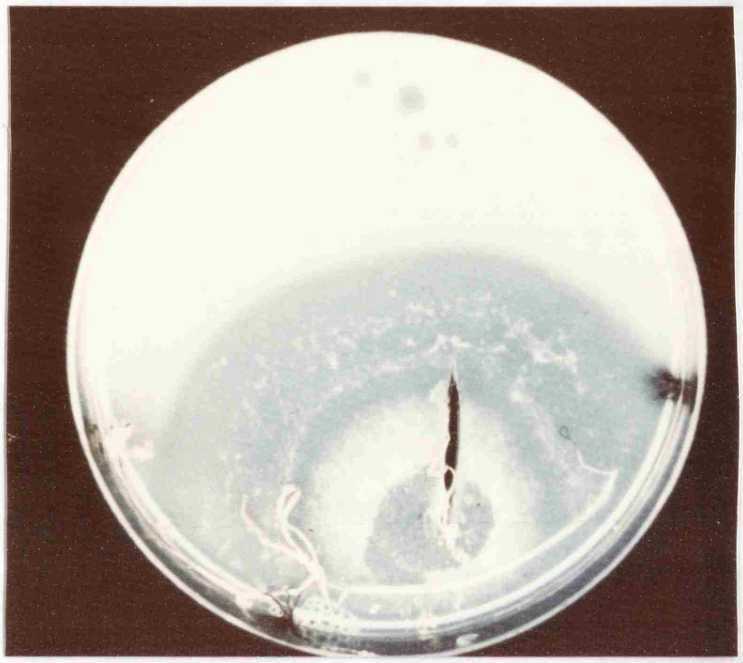
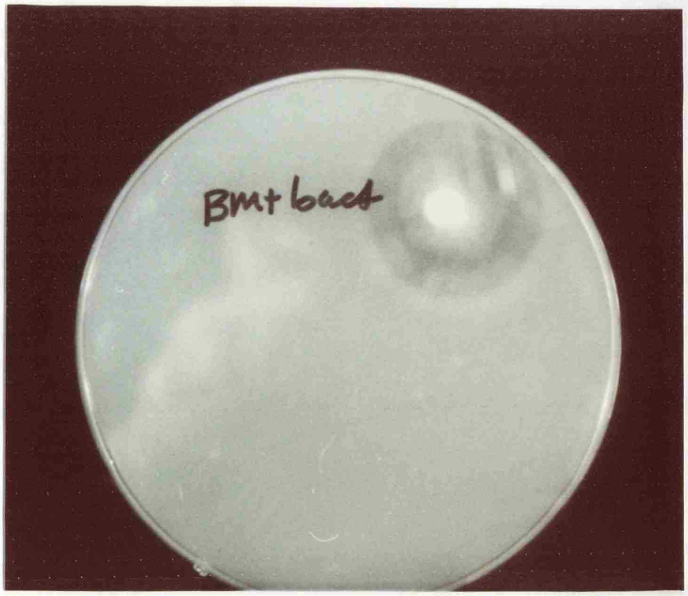


Figure 22

Degradation of autoclaved thermophilic bacterial cells by Agaricus bisporus



more rapidly than A.bisporus (Fig.22). Cellular breakdown by Chaetomium olivaceum was very restricted and could not be recorded photographically. Only A.bisporus could degrade non-autoclaved thermophilic bacteria and here digestion and growth was poor. However, the growth of A.bisporus on thermophilic fungal cells was more pronounced. A fungal isolate of Humicola sp. able to grow rapidly at 45°C, and slowly at 25°C, when used as a growth substrate was overgrown by A.bisporus (Fig.23). Attempts to reisolate this Humicola sp. from agar beneath A.bisporus mycelium (after 21 days) were unsuccessful whilst areas not colonised by A.bisporus yielded viable Humicola sp. mycelium. Clearing in the opaque agar did not occur (possibly because of the use of too much Humicola sp. mycelium) but the thermophilic mycelium beneath A.bisporus was much lighterin colour than the control. Growth of A.bisporus on viable cells of thermophilic actinomycetes was not recorded. Thus A.bisporus was able to grow on and possibly degrade quiescent cells of thermophilic fungi and bacteria, a property which may be linked with reductions in microbial numbers in spawned compost noted above. Organisms which possess the enzymes necessary to degrade and utilise a wide range of carbohydrate polymers and monomers occurring in a particular substrate, such as compost, would have a competitive advantage over other micro-organisms.

Figure 23

Growth of A.bisporus (white area) on a thermophilic Humicola sp. supplied as the major carbon and nitrogen source.

Numerous soil micro-organisms are known to possess the capacity to lyse microbial cell walls. Certain bacteria, Pseudomonas sp. (Mitchell and Alexander 1963), Arthrobacter sp. (Morrisey, Dugan and Koths (1976), and actinomycetes such as Streptomyces sp. (Ballesta and Alexander, 1972; Chu and Alexander, 1972) have been shown to degrade cell walls of fungi. Although Webley and Jones (1971) reported that certain soil micro-organisms may lyse fungi, few studies have been made on the ability of fungi to lyse other soil micro-organisms. A recent study of the interactions between A.bisporus and the pathogenic fungus Mycogone perniciosa has revealed that both fungi can produce enzymes capable of lysing viable cells of the mushroom (Vincent-Davies 1971) thus the mushroom must be capable of synthesising the enzymes necessary for hyphal wall degradation. Factors affecting the fungus-fungus interaction have recently been reviewed (Barnett and Binder 1973) and the parasitism of soil fungi by a number of basidio-mycetes has been examined (Griffin and Barnett 1967).

5.5 Enzyme production by A.bisporus

The ability of A.bisporus to degrade a wide range of naturally occurring carbohydrates has been known for some time (Styer 1930; Treschow 1944; Bohus 1959) and the suitability of a wide range of nitrogen containing compounds for mushroom growth in vitro is also known (Bohus 1959). The ability of

A.bisporus to degrade and utilise other naturally occurring substrates, of plant and microbial origin, was examined in an attempt to determine the acceptability of potential nutrient substrates in compost.

A range of carbohydrate polymers and monomers likely to occur in compost was incorporated into basal medium, supplemented with 0.02% w/v L-asparagine and inoculated with A.bisporus mycelium. Substrates (Table 17) were added at 0.5% w/v.

After 24 days incubation the dry weight of mushroom mycelium was determined and culture filtrates analysed for reducing sugar, (Nelson 1944; Somogyi 1952), the presence of which was taken as a positive indication of enzyme degradation of the substrate. If no reducing sugar was detected then an assay for enzyme in the culture filtrate was employed. Culture filtrate (4 ml) was mixed with 1 ml substrate (5.0 mg/ml) and 1 ml phosphate buffer (0.05m), pH 6.0, and incubated at 37°C for 1 hr. (Skujins, Potgieter and Alexander 1965). After incubation the reaction mixture was assayed for reducing sugar as above. Chitinase was assayed as described by Skujins et al (1965) using colloidal chitin as substrate.

The mycelium of A.bisporus grew well on a range of substrates supplied as carbon sources (Table 17). The detection of reducing sugar in culture filtrates with or without prior incubation in buffered substrate suggested that a wide range of carbohydrate degrading

Table 17

Mycelial growth and enzyme production by A.bisporus on simple and complex carbon in vitro

Substrate	dry weight (mg/ml)	enzyme production
xylan	12.4*	+
laminarin	8.3	+
araban	8.0	+
trehalose	7.9	-
galactan	7.1	+
pustulan	6.9	+
cellobiose	6.8	NT
glycogen	5.6	-
glucose	4.6	NT
cellulose	5.0*	C_x -, C_1 -
CM Cellulose	4.5	+
starch	4.5	-
xylose	4.2	NT
dextran	3.4	+
inulin	2.7	+
mannan	1.1	-
chitin	12.8*	-
none	1.4	-

* includes original insoluble material.

Enzyme production: positive detection (+), negative (-), not tested (NT)

enzymes may be produced by A.bisporus. Of the enzymes necessary for degradation of fungal mycelium (c.f. Bloomfield and Alexander, 1967; Ballestra and Alexander, 1972; Laborda, Garcia-Acha and Villenueva, 1974) A.bisporus produces β 1 → 3 glucanase (on laminarin) and β 1 → 6 glucanase (on pustulan). However, evidence was not obtained for chitinase production by A.bisporus, even though Vincent-Davies (1971) and Wood (1973) claim to have demonstrated chitin degradation in vitro. No reason can be given for the lack of chitin degradation recorded here.

Hemicellulose obtained from larch wood xylan (Sigma Company) was readily degraded by the mushroom thus confirming the earlier findings of Styer (1930), Bohus (1959) and Hashimoto, Isobe and Takahashi (1967). Carboxy methyl cellulose (CMC) was also readily degraded, especially in buffered substrate, suggesting the production of a CMCellulase (C x enzyme). However, on ashless flock cellulose (Whatman Co.) no prehydrolytic cellulase enzymes (C_1) were detectable. The production of cellulase enzymes by A.bisporus is discussed below.

The growth experiments described above were repeated using the same basal medium but supplemented with D-glucose (1.0% w/v) and 0.1% w/v nitrogen source. The range of nitrogen substances used (Table 18) included some of the compounds which might result from degradation of bacterial cell walls (Salton 1964)

Table 18

Growth of A.bisporus on a range of nitrogen containing compounds in vitro

Compound	Dry weight mycelium (mg/ml)	
gelatine	3.94	a
casein hydrolysate	4.10	a
asparagine	2.56	a b
$(NH_4)_3$ PO4	3.79	a
$(NH_4)_2$ SO4	3.05	a
$NaNO_3$	2.62	a b
chitin	2.80*	-
diamino pimelic acid	2.20	c
N-acetyl glucosamine	2.99	a b
glucosamine HCl.	2.94	a b
ATP	2.67	a b
AMP	2.82	a b
adenine	1.20	c
guanine	2.04	c
cytosine	2.51	b
uracil	2.53	b
none	1.96	c
DNA	+	
RNA	+	

Numbers followed by a similar letter are not significantly different at p = 0.05 (Duncan Multiple Range test).

* includes insoluble chitin

or chitin. After 18 days incubation the dry weight of mushroom mycelium was assessed.

Protein and inorganic ammonium salts were the most suitable nitrogen sources for A.bisporus (Table 18) confirming the earlier findings of Styer (1930) and Bohus (1959). Amino acids and amino sugars were also readily utilised. Uracil and cytosine were more effectively used than adenine, quanine or chitin. By using DNAse and RNAse detecting agars (Oxoid) it was possible to demonstrate that these nucleic acids may be degraded also.

The availability of these carbon and nitrogen sources to other fungi was tested by repeating the experiments using Ch.olivaceum, S.stemonitis, T.roseum, and C.fimentarius. Four carbohydrate and six nitrogen sources were tested with these fungi.

Whereas A.bisporus was able to utilise all test carbohydrate sources other fungi were restricted on one or more of these substrates. (Table 19). All fungi grew well in media containing gelatine or asparagine as nitrogen sources, and only Trichothecium roseum was restricted by other nitrogen sources (Table 20). These results would indicate that, whilst A.bisporus may have a competitive advantage over other fungi in carbohydrate supply, no such advantage exists for nitrogen supplies. However, these substrates were utilised when supplied in a simple, free form. The relative rates of use of such substances whilst still incorporated into more complex substrates remains to be clarified.

Table 19

Comparative growth of fungi on different carbon sources (mg/ml)

Fungus	Substrate (carbon supply)				
	CMC	xylan*	dextran	laminarin	none
A.bisporus	4.5	12.5	3.4	8.3	1.4
Ch.olivaceum	3.5	3.6	0.2	2.9	0.03
S.stemonitis	0	0.8	0	0.2	0
P.versicolor	0.02	1.3	0.1	1.1	0.1
S.commune	0.2	0.5	0.3	4.6	0.1
T.roseum	0.5	4.9	0.04	2.4	0.3
C.fimentarius	0.5	3.2	0.8	2.1	0.4

* includes undissolved xylan

All incubated for 9 days at 25°C except C.fimentarius (14 days) and A.bisporus (24 days)

Table 20

Comparative growth of fungi on different nitrogen sources (mg/ml)

Fungus	Substrate (nitrogen supply)						
	asparagine	gelatine	DAPA	glucosamine	N-acetyl glucosamine	cytosine	none
A.bisporus	3.9	2.6	2.9	2.2	2.9	2.5	1.0
T.roseum	2.9	3.8	0	0	0	0	0
S.stemonitis	4.9	1.4	0.9	1.8	1.8	0.6	0
S.commune	2.3	2.4	1.0	1.4	1.6	1.2	0.8
P.versicolor	2.7	4.9	1.1	1.1	1.5	0.8	1.0
Ch.olivaceum	3.2	1.9	1.2	1.4	0.9	0.9	0
C.fimentarius	4.1	4.0	1.9	0.9	1.2	1.1	0.8

All incubated for 5 days except A.bisporus (18 days).

5.6 Utilisation of native cellulose by A.bisporus

Styer (1930) demonstrated that the cultivated mushroom could grow on a wide range of carbon sources including xylan, protein, 'humus', and 'ligno-complexes', but not cellulose. In their early studies Waksman and Nissen (1931) considered that the mushroom utilised lignin, protein, and to a lesser extent hemicellulose and cellulose from compost. Later, cellulose was implicated as being one of the major carbon sources in compost for mushroom growth (Waksman and McGrath 1931; Waksman and Nissen 1932). However, long incubation periods were used in these studies and no assessment was made of the synthesis of materials such as extracellular bacterial polysaccharides (Stanek 1972), or mycelial glucans (Webley and Jones, 1971) synthesised by many fungi or the mushroom (Treschow 1944; Vincent-Davies, 1971).

Utilisation of pure cellulose was claimed for Psalliota (Agaricus) bisporus (Bohus 1959) but the experimental techniques involved were not adequately described. Proof of degradation of native cellulose by A.bisporus would require a demonstration of the production of the necessary enzymes. Although Turner (pers. comm. 1973; Turner et al 1975) and Wood (pers. comm. 1975) have detected CMCellulase (C_x) from A.bisporus the prehydrolytic enzyme (C_1) necessary for the complete degradation of native cellulose has not been demonstrated. The ability of A.bisporus to produce

cellulose degrading enzymes was examined in the present study, a number of different experimental techniques being used.

The cellulose clearing method (Rautela and Cowling, 1966) was modified as follows; the basal growth medium (General Materials and Methods) was supplemented with 0.5 g/l cellulose, 0.2 g/l L-asparagine, and agar. Ashless flock (Whatman), filter paper (Whatman No.1), absorbent cotton wool (Robinson & Sons Ltd., Chesterfield), microcrystaline cellulose (Sigmacell, Sigma Co.), and wheat straw were milled through a 0.2 mm sieve (Glen Creston Ltd., Stanmore, England), before being used as cellulose substrates. The cellulose was maintained in suspension by agitation until the agar had solidified. After inoculation and incubation at 25°C for up to 4 weeks areas of clearing, and extent of mycelial growth into the agar were noted. Chaetomium olivaceum was included in the experiment for comparison.

No clearing was noted around the mycelium of A.bisporus but growth into agar had occurred. The clearing associated with the mycelium of Ch.olivaceum confirmed the validity of the assay procedure (Table 21). Ashless flock appeared to be the most acceptable form of cellulose for degradation by Ch.olivaceum (Table 21). Cellulase production in other compost saprophytic fungi was measured using ashless flock as substrate, the fungi tested being listed in Table 22.

Table 21

Effects of A.bisporus and Ch.olivaceum growth on cellulose agar.

Cellulose source	A.bisporus		Ch.olivaceum	
	mycelial growth (cm)	clearing* (cm)	mycelial growth (cm)	clearing* (cm)
microcrystaline	0.6	0	0.2	0.3
ashless flock	0.5	0	0.3	0.4
cotton wool	0.5	0	0.2	0.3
filter paper	0.5	0	0.1	0.3
straw	0.5	0	0.2	0.2

* depth of clearing measured from agar surface

Table 22

Relative frequency of cellulase production in saprophytic fungi as indicated by clearing in cellulose agar

Test fungi	Mycelial growth (cm)	Clearing in agar (cm)
Trichoderma sp.	0.1	0.3
D.dendroides	0.2	0
S.stemonitis	0.4	0
Fusidium sp.	0.1	0
S.thermophile	0.2	0.1
Ch.olivaceum	0.3	0.4
C.fimentarius	0.2	0.1

Results after 10 days incubation

Three fungi sometimes found growing in compost produced clearing in cellulose agar viz. Sporotrichum thermophile, C.fimentarius and Ch.olivaceum (Table 22). Stysanus stemonitis, a common compost inhabitant, did not degrade cellulose suggesting that synthesis of cellulase enzymes is not a prerequisite for colonisation of compost.

In a further attempt to detect cellulase production by A.bisporus the fungus was grown in a liquid medium devised by Petterson, Cowling and Porath (1963). This contained (g/l) NH_4HPO_4, 2.0; KH_2PO_4, 0.6; K_2HPO_4, 0.4; $MgSO_4.7H_2O$, 0.5; and minor additions (mg/l) $CaCl_2$, 55.0; $ZnSO_4.7H_2O$, 4.4; $MnSO_4.4H_2O$, 5.0; and (µg/l) thiamine hydrochloride, 1.0. Ashless flock (5.0 g/l) was used as a carbon source. The liquid medium was autoclaved as described and dispensed into 9.0 cm dia. glass Petri dishes (25 ml/dish) and multipoint inoculated with A.bisporus or six other test fungi. After incubation for 21 days, A.bisporus, or 10 days for other test fungi, a visual assessment was made of growth. Culture filtrates were assayed for C_x enzyme using carboxymethyl cellulose as substrate (Reese and Mandels 1963), and for C_1 enzyme using ashless flock (Stutzenberger 1971).

Some evidence was obtained for C_x enzyme production by a D.621 strain of A.bisporus on ashless flock, but C_1 enzymes could not be detected (Table 23). In contrast Polystictus versicolor and Ch.olivaceum produced both C_x and C_1 enzymes. Although

Table 23

Production of cellulase (C_x and C_1) by fungi growing in a defined liquid medium with ashless flock as a source of cellulose.

Test fungi	Growth (visual estimates 0 = no growth; 5 = max.growth)	Cellulase (units)*	
		C_x	C_1
Ch.olivaceum	4	3.7	1.4
P.versicolor	5	15.6	0.5
S.thermophile	3	13.6	0
T.roseum	2	6.2	0
S.commune	5	8.5	0
D.dendroides	0	0.7	0
S.stemonitis	1	0.4	0
A.bisporus	1	1.1	0

* 1 unit enzyme taken to be sufficient to release 1 μg reducing sugar/ml/hr at 37°C.

Schyzophyllum commune and Sporotrichum thermophile grew well and formed C_x enzyme, the C_1 enzyme was not synthesised.

Thus no conclusive evidence was obtained for native cellulose utilisation by A.bisporus. Both A.bisporus and Stysanus stemonitis may grow in compost at different times but neither possess an easily demonstrable C_1 cellulolytic capacity. Conversely Ch.olivaceum and C.fimentarius, both of which grow actively in poorly prepared compost, produce active C_x enzymes, but only the former produces a C_1 enzyme. This underlines the fact that a C_1 enzyme is not necessary for growth of organisms, including the mushroom, in compost.

The suggestion that the nutrition of A.bisporus might change at initiation of sporophores from one of 'nitrogen-rich lignin-humus complex' degradation to one of cellulose degradation during cropping (Gerrits et al 1965) was next considered. If this suggestion is correct then the inability to demonstrate growth of A.bisporus on native cellulose in the present study might be due to assaying at an unfavourable point in the reproductive cycle of this fungus, i.e. reproductive rather than vegetative growth might be the cellulose utilising form.

The method used to initiate A.bisporus and produce sporophores in sterile culture (Long & Jacobs 1974) was modified using the principles of the 'Halbschalentest' (Eger 1959). One side of a divided Petri dish was

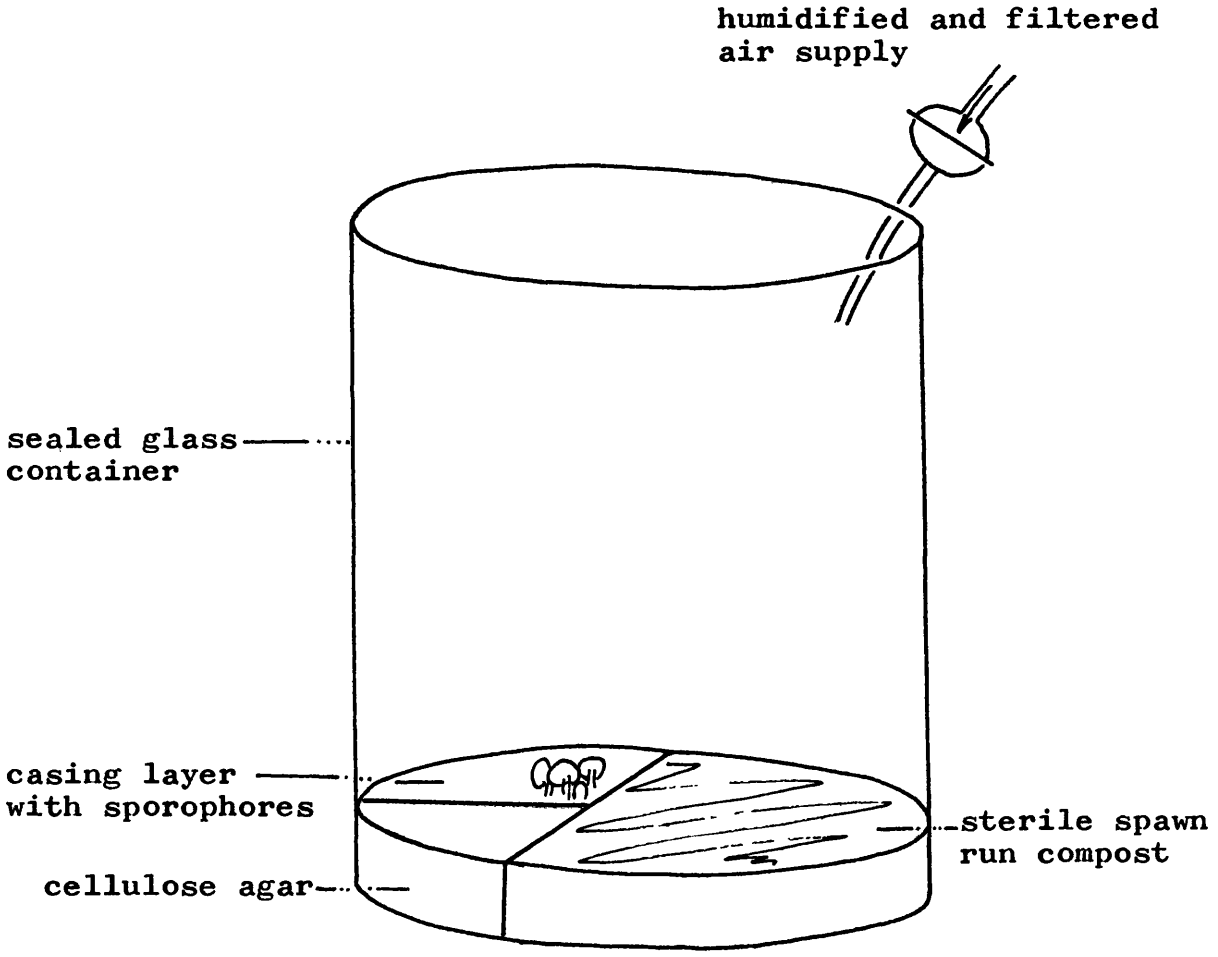
filled with compost previously dried and milled through a 0.2 mm sieve (Glenn Creston, Stanmore). The empty half was divided into two with a close fitting glass divider and one section filled with sterile casing material plus 25% w/w charcoal (B.D.H. Ltd., activated for gas absorption). Dishes were placed in a glass container (Fig. 24) with an air supply incorporating an absolute filter (Microflow Ltd.) and sealed with aluminium foil. After autoclaving the remaining section of the dish was filled with molten cooled (45-50°C) agar containing ashless flock (0.5% w/v). The compost was inoculated with A.bisporus and incubated at 25°C. After colonisation of compost, and 1 cm of casing and agar, air was flushed through the system at 10 ml/min. Fructification failed to occur even after 28 days.

The method was repeated using a bacterial suspension to induce initiation. The suspension was obtained by adding 1.0g casing material to 250 ml nutrient broth (Oxoid) and incubating at 25°C for 24 hr. Equal volumes (0.5 ml) were added to the casing material in each plate and incubated as described.

Fructification occurred after 14-20 days incubation. Sporophores (2-3 cm diameter) were removed aseptically. During mycelial growth and sporophore development sections of agar containing cellulose and mycelium were removed, stained in cotton blue (0.05% w/v) in lactophenol and examined microscopically for signs of cellulose degradation. It was reasoned that if

Figure 24

Modification of the Halbschalentest (Eger, 1959) for the production of mushroom sporophores under semi-sterile conditions.



cellulose acts as a major nutrient source during sporophore production then the addition of supplementary cellulose might result in degradation of this material. At no stage was cellulose degradation in the agar noted.

After extensive investigations during the present study it has not been possible to demonstrate native cellulose utilisation, or C_1 cellulase production by A.bisporus. It is probable that after the high microbial activity associated with peak heating there would be little readily available cellulose remaining, except that in close association with lignin. The types of enzymes required to utilise this substrate remain to be demonstrated but it is possible that the C_x enzyme is sufficient. Unfortunately, using this experimental technique sporophore development was restricted. More extensive growth of sporophores might have resulted in detectable cellulose degradation. Recently Turner et al (1975) demonstrated an upsurge in C_x cellulase production in compost at sporophore initiation. It is evident from the present results that degradation of native cellulose is not a prerequisite for mycelial growth or initiation and sporophore production.

5.7 Degradation of polysaccharides in compost

During composting of plant residues polysaccharides are readily degraded (Waksman and Reneger, 1934; Gerrits et al, 1965; Chang, 1967) with the concurrent synthesis of microbial cells and their by-products. Analytical procedures have been devised to detect quantitative changes in plant polysaccharides through composting of straw (Waksman and Stevens, 1930; Muller, 1965; Chang, 1967) but none consider the possible contribution of microbial polysaccharides produced during composting. A number of reports describe analyses of microbial polysaccharide in soil (Forsyth and Webley, 1949; Acton, Paul and Rennie, 1963; Keefer and Mortensen, 1963; Martin, 1971) but the presence of such materials in compost has not been considered.

Stanek (1972) suggested that microbial extracellular polysaccharide may act as a nutrient source for A.bisporus, this being utilised 4-7 times more effectively than glucose. This was reconsidered in the present study in an attempt to define possible substrates for mushroom growth in compost.

An isolate of a thermophilic bacterium (B_1) was cultured in mineral salts medium, low in available nitrogen, and extracellular polysaccharide isolated by acetone precipitation (Wilkinson, Dudman and Aspinall, 1955). This polysaccharide was washed three times in distilled water and reprecipitated with acetone on each occasion before being freeze dried. Basal medium

(general materials and methods) was supplemented with 0.5% w/v polysaccharide and 0.01% w/v L-asparagine, and multipoint inoculated with mycelium of A.bisporus or other test fungi. Dry weight was determined after noted incubation periods and compared with growth on glucose.

Three types of growth response were observed (Table 24) one group of fungi grew 4-6 times more rapidly on polysaccharide than on glucose; another grew more rapidly on glucose, whilst a third showed no preference for substrate. It is interesting to note that the three fungi often found growing in compost, A.bisporus, Ch.olivaceum and C.fimentarius, all preferred polysaccharide as a substrate. These results confirm and extend those of Stanek (1972) and suggest another possible substrate for the mushroom.

An attempt was made to determine the nature and origin of the major polysaccharide fractions in compost and to follow the quantitative changes in these caused by mushroom growth. Because the analytical techniques available fail to differentiate between materials of plant or microbial origin the initial quantitative procedures were supplemented with qualitative assays.

Extracellular polysaccharides have been extracted from soil in an impure form using a range of solvents (Swincer, Oades and Greenland, 1969) the most efficient of which is 0.5N NaOH. A large quantity of humic material may be co-extracted with this solvent and the separation of this from polysaccharide has proved difficult.

Table 24

Comparative growth of fungi on glucose and a bacterial extracellular polysaccharide.

Organism	Incubation (days)	Dry weight (mg.) glucose	polysaccharide
A.bisporus	21	4.4	25.2
C.fimentarius	4	0	5.4
C.olivaceum	4	1.2	4.2
D.dendroides	4	0.6	5.0
T.roseum	4	2.3	0.8
P.chartarum	4	3.9	1.5
Penicillium sp.	4	1.8	0.5
Fusidium sp.	4	3.4	4.3
V.malthousei	4	4.2	4.7

However, acid washed animal charcoal (Forsyth 1947) and gel filtration (Barker, Finch, Hayes, Simmonds and Stacey 1965; Swincer et al 1968) have successfully been used for this separation. The disadvantages of coextraction and possible alkaline hydrolysis of humic acid (Bremner 1954) have been outweighed by the efficiency of extraction. This efficiency has been increased by treating soil with 1.0N HCl prior to alkaline hydrolysis (Swincer et al 1968). Polysaccharide has been separated from the fulvic acid fraction of soil by repeated acetone precipitation (Rennie, Truog and Allen 1954). This polysaccharide was referred to as microbial 'gum' because of its sticky and gelatinous consistency in concentrated solution.

No attempts to isolate microbial polysaccharide from mushroom compost have been reported. Analyses of cellulose and hemicellulose (Waksman and Stevens 1930; Gerrits et al 1965; Chang 1967) in compost have employed techniques likely to extract polysaccharides of plant and microbial origin, though the occurrence and significance of the latter materials in compost has not been considered. Whilst recognising the limitations of extraction procedures these techniques may provide valid data on quantitative changes in compost carbohydrates especially if supported by data on qualitative changes in these fractions.

An extraction procedure was devised to examine changes in polysaccharide levels in compost during spawn run and cropping, and the major polysaccharide fractions

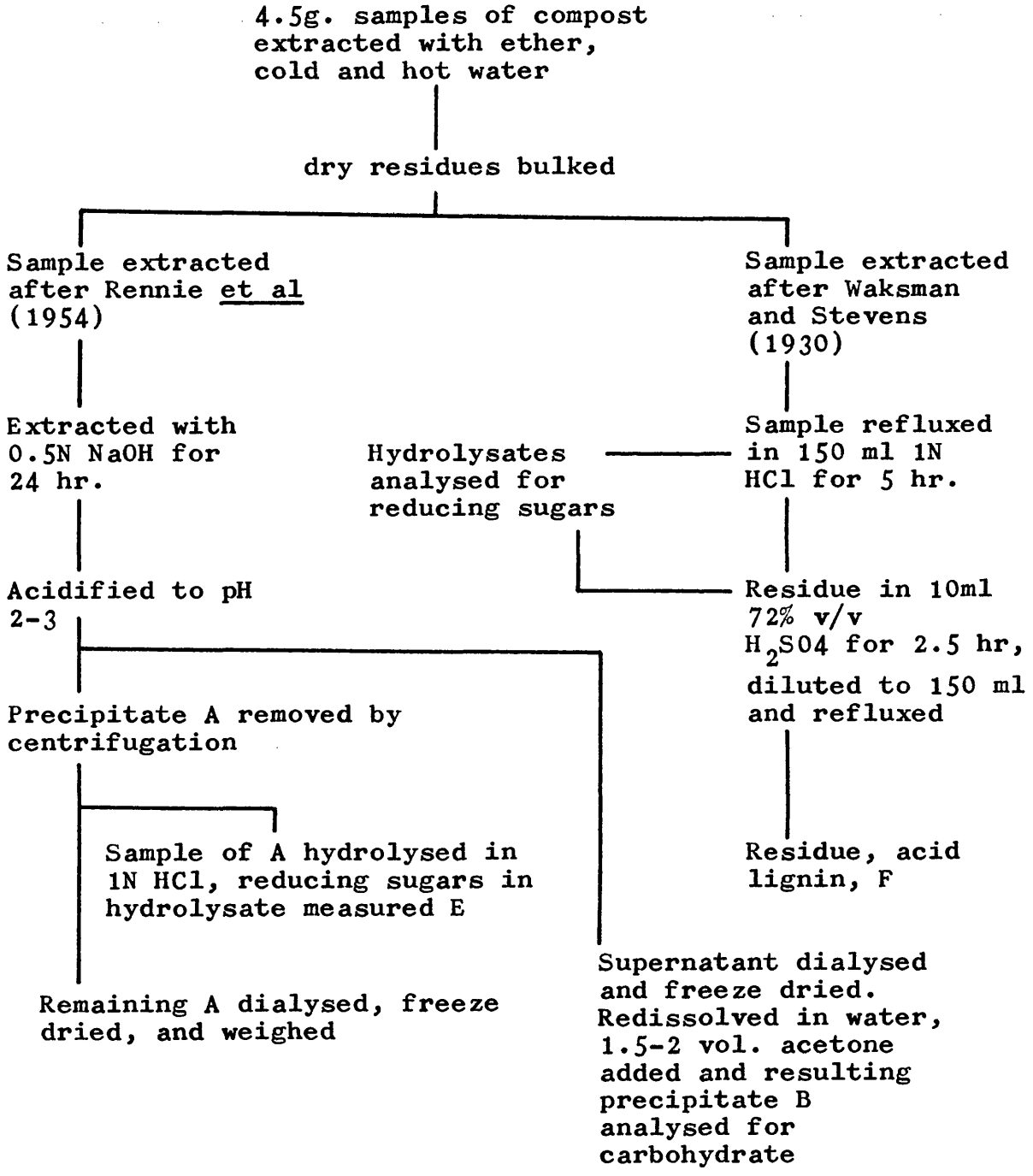
analysed qualitatively in an attempt to determine their origin, i.e. whether they were of plant or microbial origin.

Compost was extracted at different stages during the cultivation of mushrooms. One kilogram samples of compost were removed from mushroom trays at 0, 14, 28 and 60 days after spawning; these were freeze dried and ground through a 0.2 mm sieve (Glenn Creston, Stanmore, England) and extracted as indicated (Figure 25) This extraction procedure was based on those of Waksman and Stevens (1930); Forsyth (1947); Rennie et al (1954); Gerrits et al (1965) and Grabbe (1972). Fresh wheat straw was extracted at day 0 for comparison.

After removal of acetone and water soluble materials the compost was extracted for polysaccharides (Figure 25). The extraction procedure (Rennie et al , 1954) used to isolate microbial polysaccharide from soil was modified for use with compost. Three gram samples of milled compost were shaken in 50 ml 0.5N NaOH on a wrist action shaker (Griffin, set at 4) for 30 min. and then repeatedly extracted in a soxhlet thimble with cold 0.5N NaOH until most coloured material had been removed. Extracts were bulked, centrifuged at 3,000 r.p.m. for 10 min. and the supernatant adjusted to pH2-3 using conc. HCl. The resulting coloured precipitate A was removed by centrifugation and a known amount dialysed against distilled water for 24 hr. and freeze dried for dry weight estimations. The remainder of A was hydrolysed under reflux with 50 ml 1N HCl for 1 hr.

Figure 25

Procedure for extraction and estimation of compost polysaccharides.

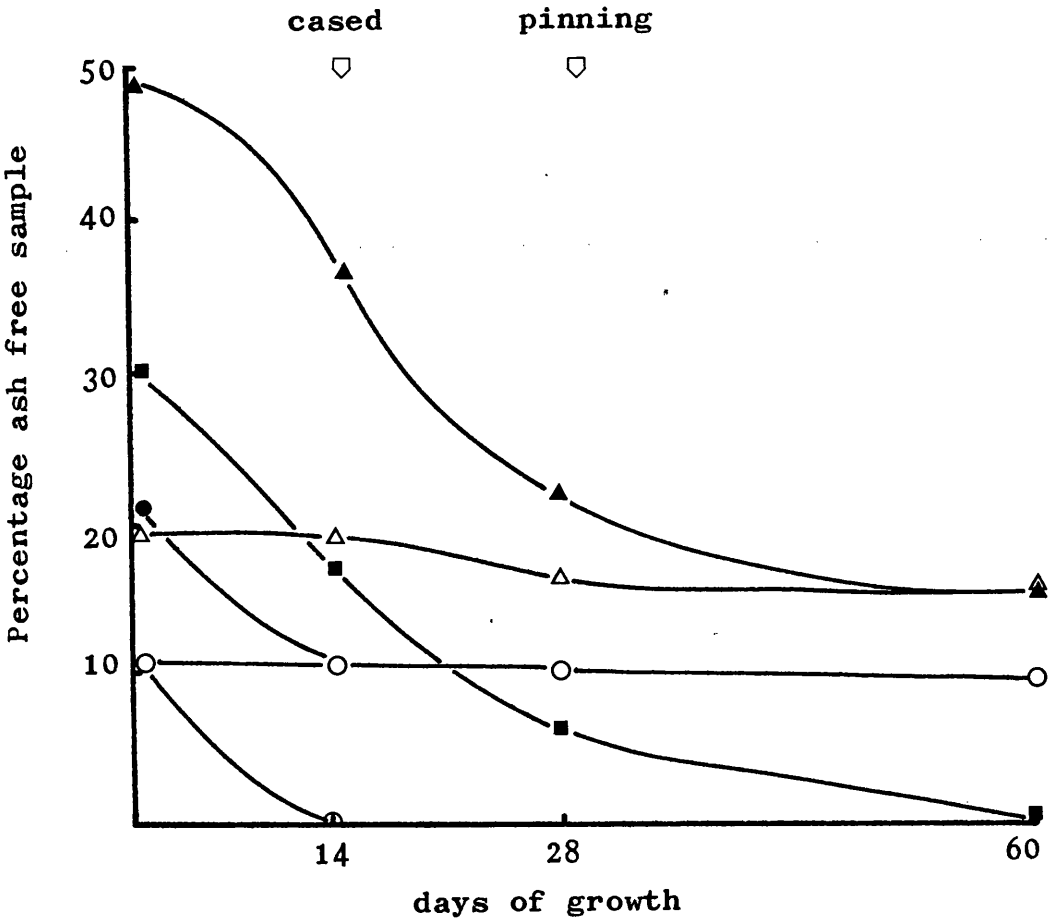


Reducing sugars in the hydrolysate were determined and taken to represent hydrolysed polysaccharide attached to the humic acid E. The alkali soluble-acid soluble supernatant was mixed with 1.5-2 volume of acetone with vigorous shaking and the resulting white precipitate collected by centrifugation. This was redissolved in 0.5N NaOH and the precipitation process repeated, dry weight of solid being determined after dialysis and freeze drying B. The alternative fractionation procedure (Waksman and Stevens 1930) involved removal of polysaccharides by two hydrolysis stages. Heating a sample of compost in 150 ml 1N HCl under reflux for 5 hr. removed a fraction of attached polysaccharide, this being estimated as reducing sugar in the hydrolysate C. The residue was soaked in 10 ml 72% w/v H_2SO_4 at 4°C for 2.5 hr, this being diluted to 150 ml and heated under reflux for 1 hr. The hydrolysate D was assayed for reducing sugar and the residue F dialysed and freeze dried.

Whereas alkali appeared to extract 49% w/w compost the equivalent fraction from fresh wheat straw represented only 15.2% (Figure 26). Reducing sugars equivalent to 30% w/w were recorded for the poly-saccharide in fraction A after alkaline extraction of compost at spawning. This value decreased rapidly during spawn growth and more slowly during cropping to a very low level. The amount of coextracted brown material, considered to be humic acid (Grabbe 1972),

Figure 26

Quantitative fractionation of compost (Rennie et al,1954)



▲ NaOH soluble, HCl insoluble precipitate

△ residue from ▲ after acid hydrolysis

■ hydrolysed material (▲ minus △)

● NaOH soluble, HCl soluble

○ residue from ● after removal of acetone insoluble material

○-⊙ acetone insoluble residue from ●

Fresh straw extracted as above yielded: (% w/w)

a) 15.2, equivalent to ▲ above

b) 12.4 " " △ "

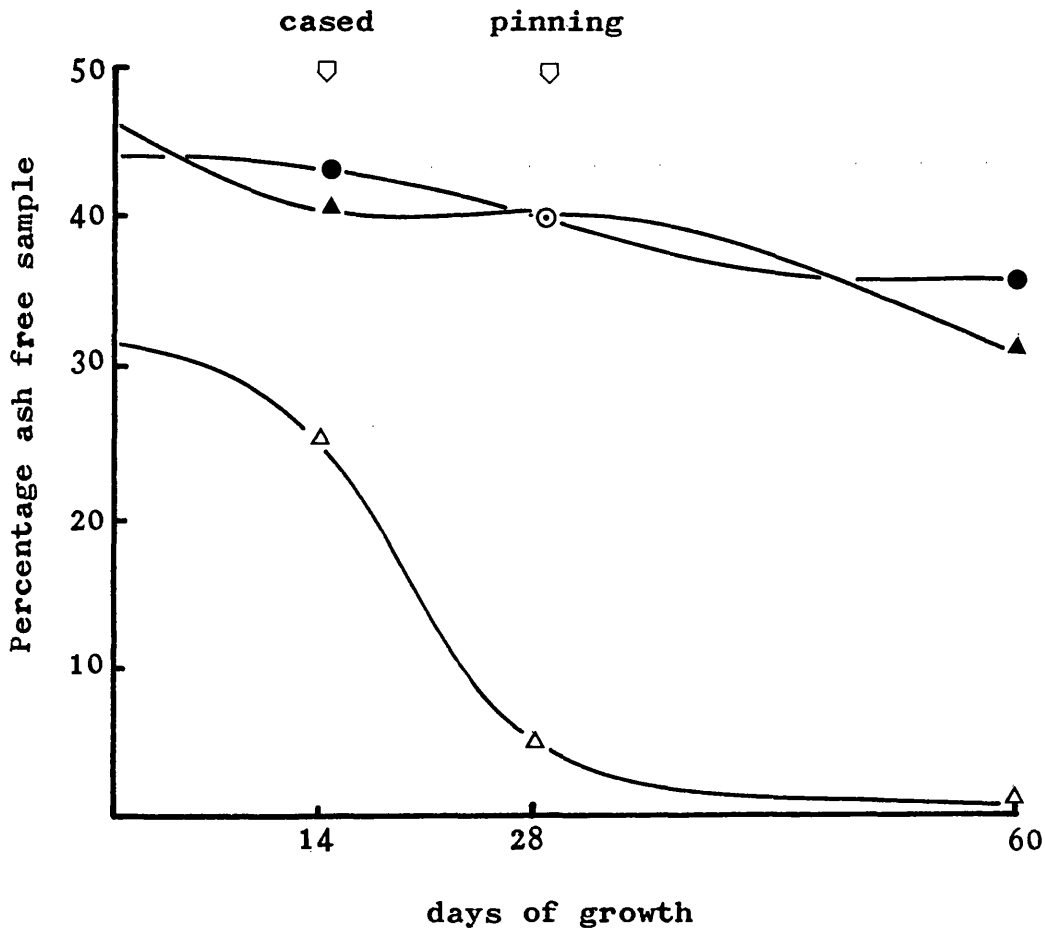
c) 2.8 " " ■ "

d) 19.2 " " ● "

e) 11.2 " " ○ "

Figure 27

Quantitative fractionation of compost (Waksman and Stevens,1930)



▲ H_2SO4 hydrolysis - reducing sugars

△ dil. HCl hydrolysis - pentose sugars

● acid insoluble residue - acid lignin

Fresh straw similarly fractionated yielded

a) 20.9% w/w, equivalent to △

b) 19.2% w/w, equivalent to ●

c) 38.2% w/w, equivalent to ▲ at day 0

remained relatively unchanged during this period (Figure 26). The alkali-soluble acid-soluble fraction contained an acetone insoluble fraction B at spawning equivalent to 10.5% w/w of sample, this rapidly decreasing during spawn run. An equivalent fraction from fresh straw represented 11.2% sample. The coextracted alkali-soluble acid-soluble acetone-soluble fraction, considered to be fulvic acid, remained relatively unchanged during incubation (Figure 26).

Results obtained by the alternative fractionation procedure (Waksman and Stevens 1930) are given in Figure 27 . Initially 16% w/w was hydrolysed from the compost sample using 1N HCl. It is interesting to note that the decrease in this fraction, following the growth of A.bisporus, closely resembled that of polysaccharides in fraction A. Hydrolysis of polysaccharides using H_2SO_4 similarly revealed a decline in levels during mushroom growth but the residue, designated as acid lignin F showed only a slight decrease over this period.

When compared with the analyses of Gerrits et al (1965) over the same period, i.e. from spawning through cropping, several differences in results are apparent. The validity of a straight line representing changes in compost constituents over 12 weeks of cropping is questionable in view of the present results. Similarly, such results (Gerrits et al 1965) must be examined closely as no attempt was made to estimate materials synthesised by micro-organisms during composting or the mushroom mycelium during spawn run. Techniques presently available

for analysing components of compost fail to distinguish clearly between materials of plant or microbial origin. Future research in this area might utilise ^{14}C labelled nutrient sources, e.g. ^{14}C-glucose or ^{14}C-acetate for closely controlled laboratory composting experiments in attempts to label microbially produced polysaccharides. Results from studies in soil suggest that this approach might yield useful data. Using ^{14}C-acetate and ^{15}N-labelled $(NH_4)_2SO_4$ McGill *et al* (1975) were able to demonstrate incorporation of labelled C and N into all organic fractions isolated, these fractions being derived through microbial metabolism.

Since polysaccharides decreased markedly during spawn run and cropping these materials were examined in more detail in an attempt to determine their origin. Polysaccharide was extracted from peak heated compost as described using 0.5N NaOH. Contaminating coloured materials were removed by passing the extract through activated charcoal filters (10 cm long x 0.5 cm. dia.) under slight pressure (Forsyth 1947). This filtrate, alkali-soluble acid-soluble, plus the acetone-insoluble fraction B were freeze dried before use. Equivalent fractions from fresh wheat straw were also prepared for comparison. The dry samples were hydrolysed by a) 20 mg. solid heated with 0.5 ml 0.1N H_2SO_4 for 30 min. at 100°C, b) 20 mg. solid heated with 0.5 ml 1N H_2SO_4 for 4 hr. (Forsyth and Webley 1949; Swincer *et al* 1969). Hydrolysates were neutralised with $BaCO_3$, desalted using anion (Dowex 2-X8 acetate cycle) and cationic resin

(Amberlite 1R-120) in 60 cm columns with a flow rate of 2 ml/min. and examined for constituent sugars using paper chromatography (Forsyth 1948) and thin layer chromatography (Stahl 1969). Hydrolysates obtained by acid hydrolysis of compost were similarly treated and examined for constituent sugars. Samples of uncomposted straw were similarly examined for comparison.

The range of sugars obtained from hydrolysates is indicated in Table 25.

Dense to moderate spots were detected for glucose, xylose, galactose and mannose in uncomposted straw fractions. Although these sugars were present in compost extracts the intensity of spots was considerably reduced. A more diverse range of sugars was obtained from compost with reduced levels of xylose, arabinose and galactose suggesting that less hemicellulose was extracted from compost than straw. Similarly, the reduced glucose detected in compost extracts probably indicates a reduction in levels of cellulose. The low levels of xylose and arabinose, and the presence of fucose, rhamnose, mannose and ribose is taken as an indication that a major proportion of the carbohydrate extracted from compost is microbial in origin (Forsyth 1954; Gupta 1967) and may be bacterial extracellular polysaccharides. This was previously suggested by the viscous consistency of a concentrated solution of isolated polysaccharide. The range of sugars detected

Table 25

Sugar composition of polysaccharides from fresh straw and compost extracts

Sugars identified by r.f. values	Substrate origin					
	compost				straw	
	NaOH soluble		acid hydrolysis		NaOH soluble	
	acid insoluble	acid soluble	HCl	H_2SO_4	acid insoluble	acid soluble
glucose	++	++	++	++	+++	+++
galactose	++	++	++	-	+++	+
arabinose	+	+	++	-	++	++
mannose	++	-	++	-	-	++
xylose	+	++	++	-	++	+++
ribose	+	+	+	-	-	-
fucose	+	++	-	-	-	-
rhamnose	+	-	+	-	-	-
unidentified	+	-	++	-	-	-

Chromatogram spots: +++ = dense; ++ = moderate; + = trace; - = none

from these polysaccharides is more varied than those obtained by Stanek (1972) from bacterial extracellular polysaccharide and may reflect a number of different sources. Whistler and Kirby (1956) and Parsons and Tinsley (1961), have extracted a wide range of sugars from soils and compost. The trace amounts of ribose and fucose recorded here are similar to those recorded by Finch, Hayes and Stacey (1971) from soils and may represent fragments from microbial cell walls, including those of A.bisporus (Vincent-Davies 1971). Uronic acids are often detected in bacterial extracellular polysaccharides (Stanek 1972) and soil (Shorey and Martin 1930) but were not detected during this study. This may be due to destruction of these materials during hydrolysis (Swincer et al 1969) or to their absence from the polysaccharide examined.

The range of sugars isolated imply that microbial polysaccharides contribute to the carbohydrate reserves in mushroom compost. The rapid fall in the levels of these materials during spawn run and cropping indicate that the mushroom is capable of degrading these carbohydrates as a nutrient source under commercial conditions.

5.8 Humic acids and lignin complexes in compost

During phase-1 of composting black materials are synthesised in the substrate (Sinden and Hauser 1953) the nature and significance of which is little understood. Sinden and Hauser (1953) considered this to arise from caramelisation of carbohydrates but Grabbe (1972)

recognised them as humic acids. In soil, humic acids are considered to be polycondensates between phenols from plant (Waksman 1916; Burges, Hurst, and Walkden 1964) or microbial origins (Mayoudon and Simonart 1958; Martin, Richards and Haider 1967) and amino acids. Amino sugars may also be incorporated (Bondietti et al 1972). Other reactions between free ammonia and di- or trihydroxyphenols (Mattson and Koulter-Andersson 1943), and amino acids and sugars at elevated temperatures (Maillard 1912; Bremner 1967) also yield black materials but these have been less extensively examined. Black materials with the same infrared spectrophotometric characteristics as soil humic acids may also be synthesised by fungal mycelium (Martin, Richards and Haider 1967 ; Haider and Martin 1970). The dark material of compost is probably produced through one or more of these mechanisms. During spawn run and cropping the colour of this material changes to a red-brown. The possible role of these dark materials in the nutrition of A.bisporus was considered in view of the suggestion by Styer (1930) that the mushroom could grow on soil humus and wood ligno-complexes as nutrient sources.

Native lignin was isolated from wheat straw using the technique of Brauns (1939), and black materials, designated humic acids (c.f. Grabbe 1972) were extracted from peak heated compost with 0.5N NaOH (Figure 25). A lignin-protein complex was prepared by mechanically grinding lignin and 20% w/w casein (British Drug Houses Ltd.) in a sterile pestle and mortar (Waksman and Iyer

1932). These materials were previously filter sterilised in solution and freeze dried before use.

Lignin, lignin-protein and compost humic acids (0.2% w/v) were suspended separately in basal medium, solidified with 1.2% w/v plain agar with or without added D-glucose (1.0% w/v) or L-asparagine (0.2% w/v), and 25 ml volumes dispensed into 9.0 cm dia. Petri dishes. Plates were inoculated centrally with 0.5 cm dia. discs of A.bisporus on peptone dextrose agar (0.3 cm thick). Diameter and density of growth, together with any clearing in the agar, were noted after incubation at 25°C.

No clearing in the lignin or lignin-protein complex agars was observed (Table 26). Clearing was noted in the humic acid agars when not supplemented with asparagine.

Growth was superior on humic acid compared with lignin, and resembled that on lignin-protein complex. However, no clearing in the opaque lignin-protein or lignin agars occurred suggesting that extensive utilisation of lignin, even in the presence of added nitrogen, did not occur. Since alkaline extraction of compost to yield humic acid probably results in co-extraction of attached polysaccharide (Figure 26) data obtained for growth of the mushroom on humic acid should be interpreted with caution. Similarly, clearing in humic acid agar does not necessarily represent degradation of this material since Flaig and Schmidt (1957) demonstrated that this could be accounted for by solubilisation of material by fungi followed by precipitation on hyphae. However, the fact that the mushroom produced a zone of clearing in humic acid agar prompted a further examination of this material.

Table 26

Relative growth of A.bisporus on lignin, lignin-protein mixture and humic acid in vitro

Substrate	Colony growth (diam. mm)	Density of hyphal growth*	Clearing in medium	Incubation time (days)
Lignin	1.8	fine	-	24
Lignin + glucose	2.4	fine	-	21
Lignin + asparagine	2.1	fine	-	21
Lignin-protein	2.0	normal	-	21
Lignin-protein + glucose	3.0	normal	-	21
Lignin-protein + asparagine	2.1	fine-normal	-	21
Humic acid	2.5	normal	+	28
Humic acid + glucose	2.9	normal	+	28
Humic acid + asparagine	2.4	fine-normal	-	28

* relative to hyphal growth on glucose-asparagine agar

The nature of the change in colour of mushroom compost during spawn run and cropping (Sinden and Hauser 1953) has received little attention. Grabbe (1972) suggested that no quantitative change occurred in the humic acid fraction of compost during this period. Results from the present study show that humic acids are not degraded to any extent by A.bisporus, although associated polysaccharides are.

The change in colour of black materials in compost was investigated as follows.

Alkaline extraction (0.5N NaOH) of compost followed by acid precipitation yielded a brown mucoid material. After dialysis against running distilled water for 24 hr. and freeze drying a shiny black, hard solid was obtained. Wheat straw, milled through a 1.0 mm sieve (supplied by Wrington Vale Mushroom Co.) was moistened with distilled water for 48 hr. at 20°C and mixed with the dry compost extract (30% w/w). Straw particles assumed a black-brown appearance, the dark material covering the straw as a paste. This was allowed to stand for 12 hr. to attain about 70-75% w/w moisture. before being inoculated with 10% w/w A.bisporus spawn (D.621 strain). A similar weight of moistened and treated straw was left uninoculated as control. After incubation at 25°C for 14 days the state of these substrates was examined.

Treated straw supported an excellent growth of A.bisporus from grain spawn and the black material assumed the typical lighter colour associated with conventional spawn run compost and, when broken open, released the

typical smell associated with such materials. Unspawned material remained free from contaminating fungi for up to 14 days, as did an untreated wheat straw control.

This change in colour of the black extract suggested a change in or degradation of the material. However, extraction of compost during a study of the attached polysaccharide suggested that the black residue remained relatively unchanged both in quantity (Figure 26) and appearance after extraction. It is very interesting to note that a material similar to conventional compost, though appearing less decomposed, may be produced in this way by using extracts from previously composted materials.

The change in colour of the extract resulting from spawn growth, particularly with regard to the rate at which it occurs, is very marked. Microbial decomposition of similarly obtained materials from soil is slow (Burges and Latter 1960; Mathur and Paul 1967). Microbial decomposition of humic acids has been associated with the ability to reduce carboxyl groups of m-hydroxybenzoic acids (Hurst, Burges and Latter 1962; Hurst 1963) and the reduction of humic acids *in vitro* has been attributed in one case to the peroxidase enzymes of a *Pseudomonas* sp. (Mishustin and Nikitin 1961). Since alkaline-soil mixtures absorb molecular oxygen from the air (Bremner 1950) any attempt to re-extract such decolourised materials from compost using NaOH would probably result in oxidation of the material and a negation of any degradative effects caused by mushroom growth. This might explain the lack of degradation of the dark materials extracted from compost and the absence of colour changes in alkaline extracts.

Any future work in this area could be performed in a nitrogen atmosphere to avoid oxygen absorption.

6.0 C:N ratios and the growth of A.bisporus

The importance of carbon and nitrogen sources and the relative proportions of each (C:N ratio) in composting and mushroom growth have been considered previously (Burrows 1951; Gerrits et al 1965). However, even knowing the C:N ratio of a compost it is impossible to determine the relative amounts of carbon and nitrogen available to A.bisporus. The nitrogen content has traditionally been considered the limiting factor both in composting and mushroom yields as evidenced by the attempts to supplement with nitrogen containing materials before composting (Lambert and Ayers 1950) and immediately before (Schisler and Sinden 1962a) and after (Schisler and Sinden 1962b) spawn run. The effects of limiting nitrogen on the growth of A.bisporus or competitor fungi have not previously been considered. During a study of wood decay Merril and Cowling (1966); Levi, Merril and Cowling (1968) and Levi and Cowling (1969) suggested that the composition of fungal mycelium was capable of great change in response to nitrogen stress. Under these conditions white rot fungi were unique in being able to continue the synthesis of diffusible cellulolytic enzymes. In view of these findings the ability of A.bisporus and C.fimentarius, two white rot fungi, Ch.olivaceum, a soft rot fungus, and a number of other saprophytic fungi to continue synthesis of enzymes under nitrogen stress conditions

was examined with a view to further elucidating factors which may be associated with specificity.

a) Effect of C:N ratio on mycelial growth: this aspect was examined using basal medium supplemented with 1.0% D-glucose and varying amounts of L-asparagine to provide C:N ratios of 10:1, 50:1, 100:1 and 1000:1 (Levi and Cowling 1969). Petri dishes containing 20 ml medium were multipoint inoculated with mycelial fragments and incubated at 25°C. After a period of incubation the mycelium was filtered into tared papers (Whatman No. 1) and dried at 85°C for 24 hr.

Effects of C:N ratios on growth of A.bisporus and other fungi in vitro is shown in Table 27. Most fungi were able to grow in a medium of low C:N ratio with freely available nitrogen but growth was restricted with increased C:N ratio in all cases. A.bisporus was affected but particularly marked growth reductions occurred with C.fimentarius and Ch.olivaceum, two fungi often found growing in compost. These in vitro results suggest that high C:N ratios play little part in specificity. However, this should be accepted with caution because of the readily available nature of the glucose and asparagine. In substrates containing more complex molecules the need to synthesise degradative enzymes might arise. Accordingly the effect of limiting nitrogen supply on cellulase (C_x) production by a number of fungi was examined.

Table 27

Growth of A.bisporus and other fungi in liquid media of increasing C:N ratios.

Fungus	Incubation (days)	Dry weight (mg/ml) C:N ratios			
		10:1	50:1	100:1	1000:1
A.bisporus	28	2.3	-	2.2	1.5
C.fimentarius	10	1.4	0.8	0.2	0
Ch.olivaceum	10	4.2	2.4	1.8	0.4
D.dendroides	10	4.6	3.8	3.3	1.2
Penicillium sp.	10	6.3	5.6	3.2	1.2
V.malthousei	10	5.7	5.2	2.5	1.8

b) Effect of C:N ratio on cellulase production: carboxymethyl cellulose, CMC (British Drug Houses Ltd.) was added to basal medium (0.5% w/v) and supplemented with L-asparagine to provide C:N ratios of 12:1, 120:1 and 1200:1. These media were multipoint inoculated and incubated as above. Dry weight of mycelium was assessed and culture filtrates assayed for C_x enzyme using CMC as substrate (Mandels and Reese 1957). One unit of enzyme was considered necessary to release 1 µg reducing sugar/ml/hr. at 37°C.

Saprophytic fungi such as Penicillium sp. and Absidia sp. were directly affected by increases in C:N ratios (Table 28). With these increases there was a reduction in mycelial growth and C_x enzyme production. Similarly, the strongly cellulolytic fungus Ch.olivaceum was affected by increased C:N ratios.

The effect of increased C:N ratios on most basidiomycete fungi was less noticeable (Table 28). Only slight reductions in dry weight and C_x enzyme production were noted in A.bisporus, P.versicolor and S.commune. However, C.fimentarius was severely inhibited by increased C:N ratios.

These results are in general agreement with those of Levi and Cowling (1969). Although only one degradative enzyme (C_x) was examined it is possible that high C:N ratios have similar effects on other extracellular enzymes produced by these fungi. Before the true significance of these observations can be ascertained the "available C:N ratio" i.e. the ratio of carbon and

Table 28

Effect of increasing C:N ratios on growth and cellulase (C_x) production *in vitro*.

Test organism	C:N ratio	Incubation time (days)	Mycelium wt. (mg/ml)	Enzyme units*
Penicillium sp.	12:1	8	7.25	3.4
	120:1	8	4.25	0.5
	1200:1	8	1.06	0.2
Ch.olivaceum	12:1	8	7.80	3.7
	120:1	8	4.35	1.3
	1200:1	8	1.21	0.3
Absidia sp.	12:1	8	0.20	0.18
	120:1	8	0.01	0
	1200:1	8	0	0
C.fimentarius	12:1	8	1.25	0.8
	120:1	8	0.18	0.4
	1200:1	8	0	0
A.bisporus	12:1	18	1.80	1.2
	120:1	18	1.45	1.6
	1200:1	18	1.25	1.5
P.versicolor	12:1	5	1.28	2.2
	120:1	5	1.28	1.6
	1200:1	5	1.41	2.1
S.commune	12:1	5	1.79	1.0
	120:1	5	1.40	1.0
	1200:1	5	1.16	0.7

* 1 unit C_x cellulase released 1 µg glucose equivalent/ml/hr at 37°C.

nitrogen in compost available to A.bisporus and/or other fungi needs to be determined. As yet there is no means of differentiating these components.

c) Effect of C:N ratio on mycelial composition; Levi and Cowling (1969) suggested that the continued synthesis of degradative enzymes by white rot fungi grown under nitrogen stress might be due to drastic changes in metabolism and composition of the mycelium in such fungi. This was considered as a possible explanation of the continued growth of A.bisporus on media of high C:N ratios.

Agaricus bisporus was grown in basal medium supplemented with 1.0% w/v D-glucose and various quantities of L-asparagine to provide media with C:N ratios of 12:1 and 1200:1. The mushroom was cultured in shallow liquid as previously described, the bulk of the mycelium being harvested by filtration during the log phase of growth. Mycelium was fractionated as described in figure 28 based on the procedures of Levi and Cowling (1969) and Herbert, Phipps and Strange (1971). Data on total nitrogen, soluble α-amino nitrogen, nucleic acids, lipids, 70% ethanol soluble (peptides and amino acids), 4.5% w/v KOH soluble (protein and nucleic acids) and insoluble residues was obtained. Deoxyribose nucleic acid (DNA) and ribose nucleic acid (RNA) were extracted using the Schneider method, modified by Herbert et al (1971), diphenylamine (Burton 1956) and orcinol (Herbert et al 1971) being used to quantify DNA and RNA respectively.

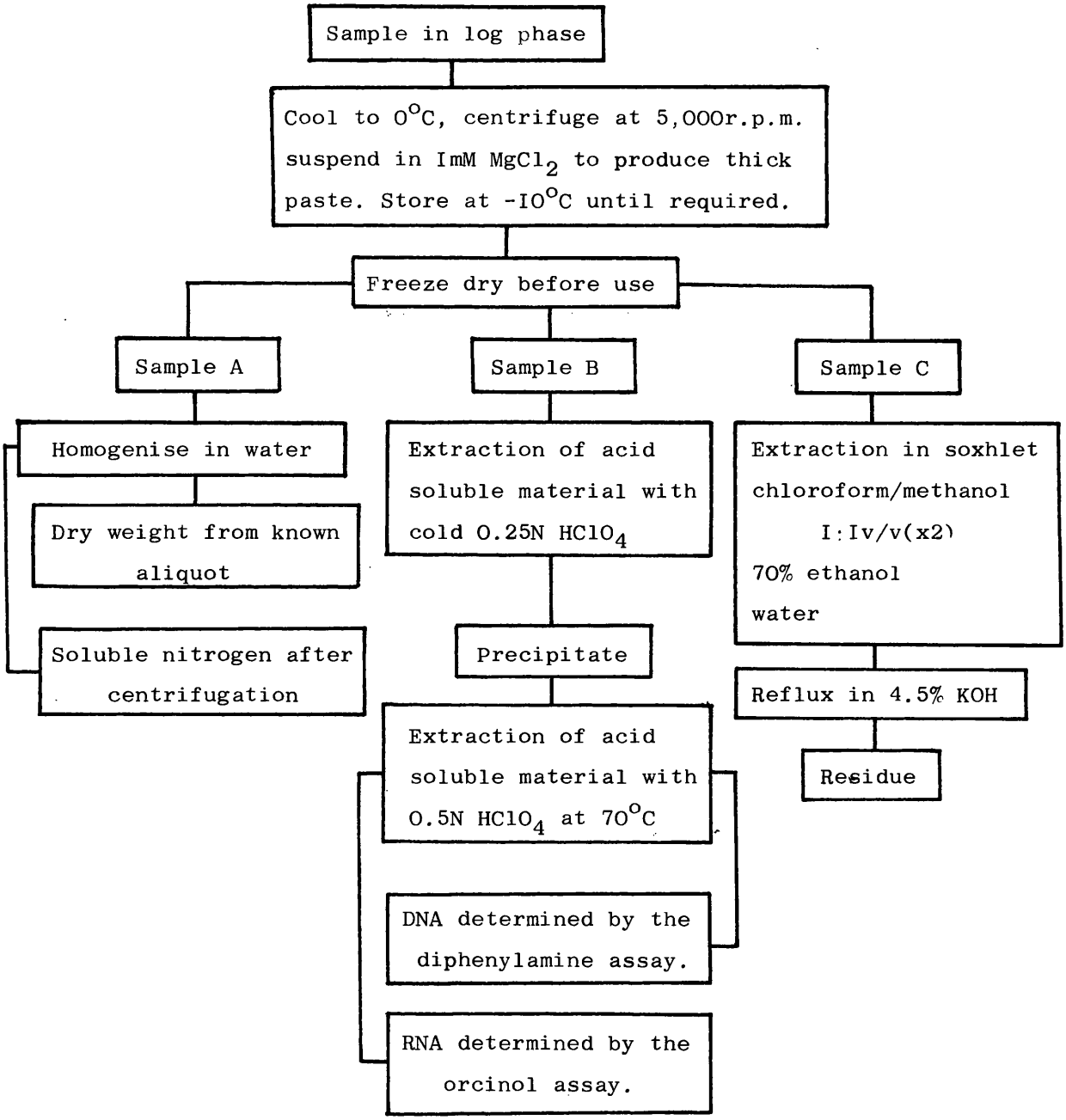


Figure 28

Procedure for the fractionation of mushroom mycelium in C/N ratio studies

The composition of mushroom mycelium grown on media of widely differing C:N ratios (12:1 and 1200:1) was markedly different as indicated by the fractionation techniques used (Table 29).

Increasing C:N ratios of media from 12:1 to 1200:1 caused a reduction in the total nitrogen of mycelium and size of all fractions other than lipid. This fraction increased from 3.2% w/w to 34.5% w/w with increased C:N ratio. The amount of nitrogen present in the nucleic acid fraction increased from 8.4% of total nitrogen at C:N ratio of 12:1 to 47.3% at C:N ratio of 1200:1. Decrease in size of fractions soluble in water, ethanol and KOH solution at the higher C:N ratio suggests that synthesis of amino acids and peptides was severely restricted. The insoluble residue was considered to be cell wall material. Preferential allocation of limited nitrogen to regions of high metabolic activity e.g. DNA synthesis, could result in reduced amino-sugar and thus chitin synthesis resulting in a reduction in cell wall residue.

These results are in general agreement with those of Levi and Cowling (1969) for changes in mycelial composition of Polyporus versicolor in vitro. In common with other white rot fungi A.bisporus is capable of continued enzyme production, e.g. C_x cellulase, under condition of nitrogen stress. Other saprophytic fungi are unable to do this and fail to grow on complex substrates e.g. carboxy methyl cellulose, in media of high C:N ratio.

Table 29

Composition of A.bisporus mycelium grown in media with C:N ratios of 12:1 and 1200:1

Medium C:N ratio	Percentage dry weight								
	Total N	Soluble amino-N	RNA	DNA	Total lipid	Ethanol Soluble	Water Soluble	KOH Soluble	Insoluble residue
12:1	3.4	2.0	1.34	0.66	3.2	69.5	12.3	0.6	11.6
1200:1	0.2	0.06	0.37	0.21	34.5	50.1	8.9	0.06	6.2

Compost is a substrate of low total C:N ratio (Burrows 1951; Gerrits et al 1965) and the significance of this work might be questioned. However, the nitrogen in compost is not freely available i.e. it is incorporated into amino acids in peptides and proteins, amino sugars in microbial cell walls, DNA, RNA etc. Much of this may be incorporated into the "nitrogen-rich lignin-humus complex" (Gerrits et al 1965). Therefore the "available C:N ratio" is unknown and it is possible that this ratio is sufficiently high to reduce or prevent growth of most saprophytic fungi in compost. Thus a selective "available C:N ratio" might represent another factor contributing to compost specificity.

7.0 'Antibiotic' production by A.bisporus

The predominance of one fungus in a particular habitat for prolonged periods is unusual but compost colonised by A.bisporus remains relatively free from other fungi, e.g. Trichoderma viride, for at least 8-10 weeks after spawning (Fordyce 1970). Numerous factors may contribute to this predominance. It was suggested above that the inability of certain fungi to grow in compost can be explained either by absence of nutrients required for spore germination, or to an inability to utilise substrates. Another factor to be considered is the production of antagonistic or antibiotic materials by the colonising organism. The possible production of such materials by A.bisporus was examined in the light of previous reports (Eger 1962; Garibova 1968; Hutchinson 1971). Volatile and non-volatile metabolites of

A.bisporus were examined to elucidate their role, if any, in compost specificity.

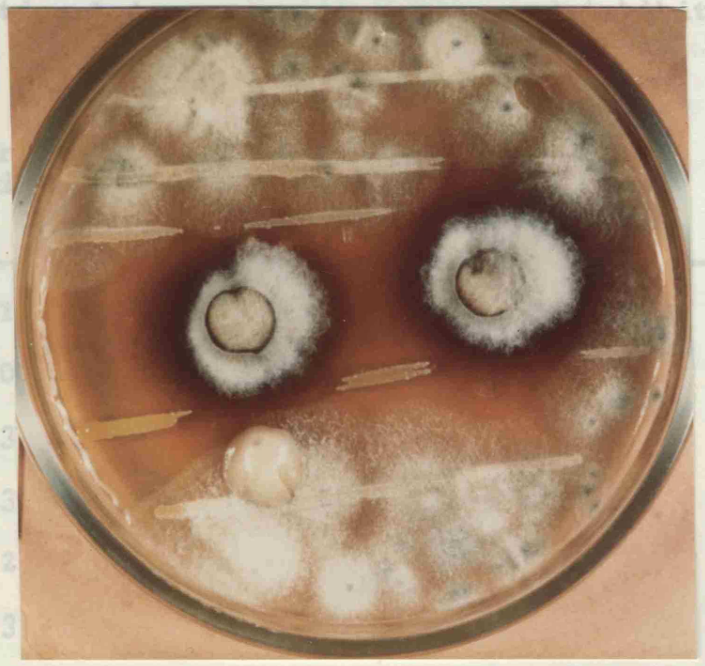
7.1 Non-volatile antagonistic materials:

Test agars (malt extract, potato dextrose, compost extract, nutrient, and B and H (Brian and Hemming 1947) were inoculated with 0.5 cm dia. discs of A.bisporus mycelium on malt extract agar and incubated at 25°C for 21 days. Seven mushroom strains were used: Pennsylvania State University (P.S.U) 310 and 318; Somycel (Som.) 22, 23 and 53; White Queen (WQ.) 102, and Darlington (D) 621. The test indicator organisms used were two bacterial isolates (supplied by Dr. G. Dutsch, Sinden-Hauser, Gossau, Switzerland) and a Penicillium sp. isolated from peak heated compost. After incubation for 10 days the test agars were streaked with test organisms at different distances from the growing mycelial front (Figure 29) and incubated further at 25°C for 5 days.

Nutrient agar and B and H agar detected inhibitor production by A.bisporus (Table 30). On these media growth of A.bisporus was reduced and inhibition was invariably associated with the production of a brown pigment in the agar. The inhibition of test organisms was not affected by pH of the media.

Figure 29

Production of antagonistic materials and brown pigment by A.bisporus on nutrient agar



(Fungi (Penicillium sp) not part of the assay)

Table 30

Relation between growth medium and inhibition of test organisms by A.bisporus

Mushroom strain	Medium				
	NA	B & H	PDA	MEA	CA
D.621	+	+	-	-	-
WQ.102	+	+	-	-	-
PSU 310	+	+	-	-	-
PSU 318	+	+	-	-	-
SOM 22	+	+	-	-	-
SOM 32	+	+	-	-	-
SOM 53	+	+	-	-	-
pH of medium	7.4	5.6	5.4	5.6	6.8

\+ indicates inhibition of two bacterial and one fungal test organisms

\- indicates no inhibition

Since NA and B & H media are high in nitrogen containing materials the significance of nitrogen in the production of antagonistic factors *in vitro* was assessed using potato dextrose agar supplemented with 0, 2, 0, 5.0, 10.0 and 25.0 g/l mycological peptone.

High levels of peptone were necessary for the production of the antagonistic effect on PDA (Table 31). Reduced mycelial growth of *A.bisporus* and a brown pigmentation in the agar were again associated with inhibition of test organisms on these media.

Table 31

Relation between nitrogen in growth medium and inhibition of test organisms by *A.bisporus*

Medium	Peptone added (g/l)	growth dia. (cm. after 21 days)	Pigmentation	Inhibition
PDA	0	3.0	-	No
PDA	2.0	3.0	-	No
PDA	5.0	2.8	+	Slight
PDA	10.0	2.82	++	Yes
PDA	25.0	1.2	++	Yes
B & H	10.0	1.6	++	Yes

\- = no pigmentation in agar
\+ = slight
++ = strong pigmentation

The ability of a range of other fungi to produce a similar antagonistic effect was tested using the same technique on B & H and NA media. The fungi tested, viz. *T. roseum*, *Fusidium* sp., *S. stemonitis*, *D. dendroides*,

V.malthousei, S.pruinosum, Ch.olivaceum, C.fimentarius and Penicillium sp. exhibited neither antagonistic activity nor pigment production.

The reaction between phenolic compounds and polyphenol oxidase enzymes to produce high molecular weight dark coloured materials is well known (Bavendamm 1928; Brown and Bocks 1963). Significantly in the present study antagonism by A.bisporus in vitro was always associated with pigment production. Therefore the possible role of the extracellular polyphenoloxidase enzyme laccase, which is produced by A.bisporus, was investigated as follows.

Potato dextrose agar was amended with 10g/l amino acid, inoculated with A.bisporus and incubated as above. The amino acids used were asparagine, glycine, phenyl alanine, alanine, tyrosine and tryptophan. After incubation the agars were tested for inhibitory materials as described and pigment production in agar noted. The extent of diffusion of laccase into the agar was determined by injecting 0.05 ml 10% w/v solution of benzidine into the agar using a 250 µl syringe (Hamilton). Ten points, 2mm to 22mm from the growing mycelial front of A.bisporus were treated. Laccase oxidises benzidine to produce a blue pigment (Dijkstra, Scheffers and Wiken 1972). Antagonism was associated with laccase and pigment production on media containing aromatic amino acids (Table 32). Although laccase diffused into agar containing non-aromatic amino acids no pigmentation or antagonism was noted. No attempt was made to isolate the

Table 32

Effect of substrate on inhibition of test organisms by A.bisporus.

Substrate	Inhibition (diam. cm)	Laccase diffusion* (diam. cm)	Pigment production
glycine	0	2.6	-
asparagine	0.2	2.4	-
alanine	0	3.2	-
phenylalanine	2.0	2.6	+
tryptophane	2.3	3.6	+
tyrosine	2.3	3.4	+

- = no pigmentation; + = pigmentation

* laccase detected using benzidine solution

antagonistic materials so the possibility that the pigmented materials were not responsible for the antagonism remains.

Since compost probably does not possess high levels of free aromatic amino acids this form of antagonism might be regarded as insignificant. However, because a whole range of aromatic phenolic compounds can be oxidised by laccase, some of which may occur in compost, the role of quinones in antagonism should not be dismissed. The antimicrobial activity of quinones has been discussed Goodman et al (1967) and the occurrence of quinones in soil investigated. No evidence for the presence of quinones in compost is available.

The concentration of nutrient in the inhibition zones was not determined and consequently no comment can be made concerning the suggestion by Hsu and Lockwood (1969) that inhibition may result from nutrient deficiency. However, it is unlikely that media so high in available nutrients as PDA supplemented with peptone, could be depleted of nutrients to such an extent as to cause inhibition in this way.

7.2 Volatile inhibitors produced by A.bisporus

Eger (1962) suggested that the mushroom mycelium growing in vitro produces volatile metabolites which may be antagonistic to other micro-organisms. An examination of the closed atmosphere over A.bisporus grown on sterile grain revealed that ethyl alcohol, acetone, acetaldehyde and ethylene may be produced (Lockard and Kneebone 1962). However, Tschierpe and Sinden (1965)

showed that ethyl alcohol was only produced under anaerobic conditions. Stauble and Rast (1971) also identified a range of volatile materials from sporophores including acetic, n-butyric and isovaleric acid; ethylmethyl ketone, isobutanol, n-butanol, isoamylalcohol, n-amylalcohol and acetic acid were detected in volatiles from basidiospores. More recently Turner, Wright, Ward, Osborne and Self (1975) have identified nine volatile hydrocarbons, as well as methyl chloride, carbonyl sulphide and carbon disulphide as products of A.bisporus in commercial compost. The suggestion that volatile materials from the mycelium may have a role in the life cycle of the mushroom (Lockard and Kneebone 1962) was confirmed when Lösel (1964) demonstrated that these materials, and particularly iso-valeric acid, stimulate germination of mushroom basidiospores.

The effect of these volatile materials on the growth and development of compost inhabiting saprophytic micro-organisms was examined using spawned compost and pure mushroom cultures. The method of assay described previously (4.3) with sealed and unsealed plates was repeated but spawned compost was substituted for peak heated compost.

The presence of growing mushroom mycelium in sterile and non-sterile compost significantly reduced ($p=0.05$) the number of fungi which developed on agar (Table 33). The numbers of actinomycetes and bacteria developing on agar over non-sterile compost colonised by A.bisporus was also significantly reduced ($p=0.05$),

Table 33

Effect of volatile metabolites from A.bisporus on development of compost micro-organisms (unsealed plates)

Organism (Numbers/g. dry weight compost)	Substrate				
	Sterile compost	Peak heated compost	Spawn run compost	Sterile compost with mushroom	LSD (p=.05)
Bacteria	46.5×10^8	43.0×10^8	4.3×10^8	36.3×10^8	16.1
Actinomycetes	21.2×10^7	22.9×10^8	1.6×10^7	14.8×10^7	8.8
Fungi	24.9×10^4	18.5×10^4	2.6×10^4	8.9×10^4	7.6

Results for sealed plates in Appendix 2.

Table 34

Effect of volatile metabolites from mushroom on growth of saprophytic fungi (plates unsealed)

Test organism	Percentage growth*				Incubation (days)
	Peak heated compost	Spawned compost 14 days	Spawned compost 21 days	Sterile compost + mycelium (21 days)	
V.malthousei	- 3.7	- 14.8	- 18.8	- 11.2	7
Penicillium sp.	- 9.8	- 18.0	- 22.2	- 16.7	6
O.fimentarium	+21.4	- 18.0	- 22.2	+ 17.2	3
C.tropica	- 2.3	0	- 2.3	0	3
Ch.olivaceum	+ 5.4	- 24.6	- 4.6	- 20.3	4
D.dendroides	+ 8.0	- 39.0	- 30.3	- 22.3	7
Fusidium sp.	+ 2.3	- 1.2	- 12.0	- 3.6	7
T.roseum	- 6.3	- 39.6	- 30.2	- 33.2	7
Cop.fimentarius	- 5.9	- 50.0	- 32.4	+ 9.1	3

* diameter of colony exposed to volatiles as a percentage of growth in normal atmosphere

Results for sealed plates included in Appendix 2.

No significant decreases ($p=0.05$) in numbers of bacteria or actinomycetes occurred with sterile compost as substrate, this may have been due to the reduced vigour of mushroom growth in such substrate.

The mode of action of these materials, i.e. the inhibition of spore germination or vegetative growth were examined using fungi as test organisms.

Nine fungi were examined for inhibition of vegetative growth by repeating the above experiment but replacing the compost suspension with a 0.5 cm. dia. disc of malt extract agar colonised by test fungi. After incubation the diameters of colonies were measured and compared with growth in normal air.

The presence of mushroom mycelium in compost caused a reduction in growth of all test fungi (Table 34). Growth of these fungi over compost alone was variable. Mushroom mycelium grown in sterile compost was also inhibitory but this was less marked compared with unsterilised substrate. Results for sealed plates are included in Appendix 2 but their value is questionable since reduction in growth might have resulted from oxygen deficiency. This was suggested by the 'rotting' smell often associated with sealed plates.

To assess the effects of these volatiles on spore germination the method was repeated, discs of test fungi being replaced on agar by a range of test fungal spores. After 24 hr. incubation over spawn run compost percentage germination and germ tube length was assessed.

Table 35

Relation between production of volatile metabolites and spore germination (in unsealed plates)

Test organism		Substrates			
		Sterile compost	Sterile compost + mushroom	Spawn run compost (14 days)	Peak heated compost
D.dendroides	%*	97.9	96.6	99.0	95.6
	g.t.l.**	1.2	0.62	0.08	0.70
T.roseum	%	98.0	99.3	76.6	98.3
	g.t.l.	0.67	0.41	0.06	0.52
Fusidium sp.	%	99.3	100	57.0	100
	g.t.l.	1.53	0.70	0.02	0.58
Penicillium sp.	%	99.0	96.6	17.0	99.3
	g.t.l.	1.41	0.34	0.04	1.01
S.stemonitis	%	100	100	0	100
	g.t.l.	0.82	0.80	0	1.12

*% = percentage spore germination (28 hr.); ** = germ tube length (mm)

Results for sealed plates included in Appendix 2.

Table 36

Inhibition of spore germination of *S.stemonitis* by volatile metabolites from *A.bisporus* strains.

Strain	Germination (%)	Germination on removal to normal atmosphere (%)
D.621	0	100
WQ.102	0	100
PSU 318	0	100
PSU 324	0	98.6
Som 22	0	100
Som 32	0	99.6
Control	100	-

Varying germination responses were observed with the spores tested (Table 35). The basidiospores of C.fimentarius and ascospores of Ch.olivaceum fail to germinate on control agars. Germination of spores of S.stemonitis and Penicillium sp. was inhibited by mushroom volatiles; in other cases percentage germination was not reduced but elongation of germ tubes was retarded. By substituting other strains for D.621 it was possible to demonstrate that inhibition of spore germination was not confined to one strain (Table 36). On removal to a normal atmosphere spore germination was not inhibited indicating that the inhibitory factor is fungistatic and not fungicidal.

Thus, inhibition of growth of saprophytic micro-organisms from compost may be caused by volatile metabolites from A.bisporus mycelium. Because the two test plates were not completely sealed oxygen deficiency is unlikely to account for this effect. Growth inhibition of micro-organisms might, if it occurs in compost, represent a method for prolonging the period during which the mushroom dominates the substrate.

The identity of the inhibitory volatiles produced by the mushroom have not been investigated. Robinson and Park (1966) implicated aldehydes, possibly acetaldehyde, as being responsible for inhibiting germination of Fusidium sp. in vitro. Hutchinson and co-workers (Glenn, Hutchinson and McCorkindale, 1966; Glenn and Hutchinson, 1969) have implicated triacetylene

as the inhibitory factor in volatiles from Fomes annosus. Recently volatiles from species of Trichoderma (Dennis and Webster 1971) have been re-examined by Tamimi and Hutchinson (1975) who concluded that ethylene played no part in inhibition, this being largely attributed to carbon dioxide levels. The role of acetaldehyde was inconclusive. Conversely acetaldehyde and ethyl alcohol have been implicated in causing increased microbial respiration in soil amended with plant materials (Griebel and Owens, 1972).

In this study pure liquid samples of the volatile compounds known to be produced by A.bisporus (Lockard and Kneebone, 1962) were examined singly in an attempt to determine the nature of the volatile materials responsible for microbial inhibition in spawn run compost.

Conidia of test fungi (S.stemonitis, D.dendroides and Penicillium sp.) were streaked onto malt extract agar in uncovered 4.0 cm. dia. glass Petri dishes placed in 9.0 cm. dia. glass crystalising dishes. These were covered with 9.0 cm. dia. glass Petri dish tops and, after sealing with masking tape (3M Brand No. 1222), volatile test liquids were added to the crystalising dish using an Agla microsyringe (Wellcome Laboratories). The quantity of test material was expressed as % v/v, i.e. volume of liquid (before volatilisation) injected into a container of known volume (Robinson and Park 1966). All dishes were incubated at 25°C, germination

Table 37

Effect of acetaldehyde on spore germination of fungi

Concentration (% v/v)	S.stemonitis		D.dendroides		Penicillium sp.	
	%	g.t.l.	%	g.t.l.	%	g.t.l.
0.002	0	0	0	0	0	0
0.0002	97.3	0.21	62.0	0.06	0	0
0.00008	98.9	0.21	0	0	0	0
0.00002	98.9	0.29	100	0.10	100	0.07
0	100	0.95	99.6	0.19	100	0.19

% germination assessed after 24 hr.

g.t.l. germ tube length (mm).

being assessed after 18 hr. Compounds tested include ethyl alcohol, ethyl acetate, acetone, and acetaldehyde at concentrations of 0.02% v/v down to 0.00002% v/v.

All compounds were inhibitory at concentrations which would probably not be encountered in compost. Acetaldehyde only was inhibitory at acceptable levels (Table 37). Concentrations of 0.00008% v/v inhibited spore germination of D.dendroides and Penicillium sp. but not S.stemonitis (c.f. Table 36). Acetaldehyde therefore appears to inhibit spores of some fungi but its reduced activity against S.stemonitis suggest that more than one compound may be involved in inhibition by mushroom volatiles.

The effect of ethylene on spore germination was not assessed but it was detected in the atmosphere over mushroom mycelium on sterile grain (Lockard and Kneebone, 1962) and more recently in commercial mushroom beds and axenic laboratory cultures (Turner et al, 1975). Smith (1973) and Lynch and Harper (1974) have suggested that ethylene might be a causal agent in soil fungistasis but this was not investigated in the present study.

In view of the reports of Tamimi and Hutchinson (1975) suggesting that CO_2 could be responsible for inhibition of this type this metabolite should also be considered as a possible inhibitor. In the present study the use of completely sealed plates, in which CO_2 could accumulate, did not result in increased inhibition from substrates other than spawn run compost.

DISCUSSION

Improved commercial practices for the control of quantities of raw materials, composting techniques and hygiene have resulted in the production of a substrate specific for the growth of A.bisporus. Relaxation of these control measures may lead to a substrate unsuitable for mushroom growth and inhabited by weed moulds, competitors and pathogens (Atkins, 1952, 1974; Sinden, 1972). These fungi may have serious implications for growers in the case of the competitive species C.fimentarius and Ch.olivaceum, or may be of nuisance value, e.g. Oedocephalum fimentarium, S.stemonitis, etc. (Duncan, 1949; Gandy, 1952; Atkins 1952, 1974). Ecological control measures may be used to exclude certain fungi from compost (Sinden, 1971) but a better understanding of the finished substrate may help avoid some of the problems encountered during composting. The purpose of this study was to determine the nature of compost specificity in peak heated compost and elucidate the mechanisms which favour the rapid growth of mushroom mycelium in this substrate.

A better knowledge of factors contributing to compost specificity might enable a process of minimal composting to be adopted for substrate production thereby conserving raw materials and reducing preparation time, labour and costs. Alternatives to straw, e.g. wood sawdust (Block and Rao, 1962) or sugar cane bagasse (Kneebone and Mason, 1972) as bulk ingredients or as supplements in composting might be envisaged.

Certain problems from weed moulds encountered during express substrate preparation (Smith, pers.comm.) might also be overcome. Since poorly prepared composts are frequently colonised by certain fungi these and other micro-organisms isolated from compost were used in this examination of compost specificity.

In spite of requirements for spore germination such as temperature, moisture, oxygen tension and pH (Lilley and Barnett, 1951; Cochrane, 1958) being satisfied in well prepared compost, the germination of spores of potential competitors and successful elongation of germ tubes failed to occur. Germination responses resembled those observed in soil (Dobbs and Hinson, 1953; Lingappa and Lockwood, 1962; Ko and Lockwood, 1967) and represent the first record of fungistasis in compost.

An explanation of specificity in terms of antibiotic or inhibitory chemical would require that these materials be extracted from compost and be present at levels sufficient to cause inhibition of micro-organisms other than the mushroom. During this study an extensive series of extraction and assay procedures failed to detect any inhibitory fractions. Although inhibition of spore germination and germ tube elongation was demonstrated by low levels of phenolic compounds no evidence was obtained to implicate these as causing fungistasis in compost. However, phenolic inhibitors should not be totally dismissed since Lingappa and Lockwood (1962) suggested that such

materials might be active in highly organic soils. Stimulation of mycelial growth of A.bisporus and S.commune by low concentrations of phenolic compounds may be similar to that recorded for Marasmius foetidus in tannic acid medium (Lindberg and Korjus, 1949) where the phenolic material caused increased glucose metabolism. Although certain soil micro-organisms are capable of degrading phenolic compounds (Henderson and Farmer, 1955) the possible role of these compounds in compost or soil has received little attention. No quantitative data was obtained for levels of phenols in compost but concentrations which inhibit spore germination in vitro probably do not exist freely in this substrate. The demonstration by Lund, Robertson and Whalley (1953) and Martin et al (1967) that soil inhabiting micro-organism can synthesise a wide range of phenolic compounds from simple precursors, glucose and asparagine, might explain the wide variety of unidentified phenolic compounds extracted from compost. Spore germination was not inhibited by wheat straw lignin or compost humic acid suggesting that these materials do not contribute to compost specificity.

One of the most rational approaches to the study of soil fungistasis was adopted by Ko and Lockwood (1967) who postulated a nutrient deficiency theory to explain this phenomenon. This was developed and extended over a number of years (Lingappa and Lockwood, 1962, 1964; Ko and Lockwood, 1967, 1970; Sneh and Lockwood, 1976) and contends that inhibition of fungal

spore germination is due to a general nutrient deficiency in the substrate and/or an induced nutrient deficiency within the spore. The mechanism by which a depleting nutrient gradient prevents spore germination is unknown. However, Sneh and Lockwood (1976) postulated that a critical concentration of soluble nutrients within the spore may be essential for germination. Though not wholly accepted (Watson and Ford, 1972) this theory enjoys widespread support and has been implicated in the inhibition of germination of chlamydospores (Adams, Lewis and Papavizas, 1968), sclerotia (Green and Ayanru, 1975), microsclerotia (Emmatty and Green, 1969) and in hyphal lysis in soil (Ko and Lockwood, 1970). Similarly fungistasis in the phyllosphere has been attributed to nutrient deficiency (Blakeman, 1975), as has bacteriostasis in soil (Davis, 1975) and inhibition of germination in some actinomycete spores (Mayfield et al, 1972). Inhibition of spore germination in certain fungi may be achieved by mechanical leaching (Ko and Lockwood, 1967; Lloyd and Lockwood, 1966). In soil this is believed to be due to microbial growth on the spore surface, a phenomenon demonstrated by Mayfield et al (1972), Old and Schippers (1973) and Old and Wong (1975). Results obtained in this study suggest that the mechanisms of inhibition of spore germination in compost follow a similar pattern. However, whereas Lingappa and Lockwood (1964) recorded increased microbial numbers after a few hours in soil supplemented with fungal spores, similar increases were not recorded in the present study. This is attributed

to the nature of the microflora in the different substrates, i.e. compost represents a pasteurised substrate with predominantly resting microbial propagules which would not rapidly enter a growth phase.

One requirement of the nutrient deficiency theory is that substrates should be low in readily available nutrients (Ko and Lockwood, 1967). Gross extraction of compost failed to provide reliable data on nutrient levels because of coextraction of intracellular nutrients from the large microbial population.

Increased microbial activity following supplementation with nutrients indicated the potential microbial activity of compost. This rapid utilisation of certain substrates resembled that recorded in soil by many observers including Stevenson (1956), Rovira (1956), Lingappa and Lockwood, (1964). Increased microbial activity in compost was correlated with reduced growth of A.bisporus suggesting that the mushroom usually grows under conditions of low nutrient availability and hence little microbial competition. The observation that the mushroom will not colonise the substrate until the correct stage of microbial succession is achieved (Garrett, 1963) is in agreement with the results of this study. Thus the data obtained from compost supports the concept that nutrient deficiency is primarily responsible for the behaviour of fungal spores in this environment. In other words

the function of composting is to provide a substrate very low in nutrients which can be readily absorbed by fungal spores.

Evidence exists for the production of volatile fungistatic materials from soil (Dobbs and Bywater, 1957; Hora and Baker, 1972a; Balis, 1973; Griffin, Hora and Baker, 1975). Inhibiting volatiles may also be produced in sterile alkaline soils (Baker and Hora, 1973) suggesting a basis for the concept of microbial and residual fungistasis (Dobbs and Bywater, 1957). The effect of volatile inhibitors may be overcome by adding simple carbon and nitrogen containing nutrients (Griffin et al, 1975). No inhibitory volatiles were detected in the atmosphere over peak heated compost during this study. However, evidence was obtained which suggests that the mushroom mycelium can produce volatile materials during the colonisation of compost; these may be important in maintenance of compost specificity (c.f. Eger, 1962). Of the volatile materials produced by A.bisporus (Lockard and Kneebone, 1962; Tschierpe and Sinden, 1965; Turner et al, 1975) which were tested in the present study only acetaldehyde appeared to be active at low concentrations. Spore germination results suggested that acetaldehyde alone could not explain the observed inhibition of microflora. Acetaldehyde has been implicated in causing inhibition of spore germination in cultures

of Fusarium sp. (Robinson and Garrett, 1969) but during a study of volatile materials from Trichoderma sp. (Tamimi and Hutchinson, 1975) the effects of acetaldehyde proved inconclusive. Other volatile metabolites from fungi have been identified and may inhibit associated microflora. Basidiomycetes may produce hydrocyanic acid (Robbins, Rolnick and Kavanagh, 1950) a compound reported to depress populations of Fusarium sp. in soil (Timonin, 1941). Triacetylene (hexa1-3-5 triyne) from Fomes annosus may inhibit spores of several fungi (Hutchinson, 1973). Thus, volatile inhibitors are not uncommon from basidiomycetes; when produced by A.bisporus they have an active role to play in prolonging specificity of spawned compost.

Another factor which might be important in maintenance of compost specificity is the interaction between the enzyme laccase and phenolic compounds in compost. Experiments in vitro suggest that the products of reactions between laccase and aromatic amino acids or unidentified materials in peptone can inhibit bacteria. Many phenolic compounds are known to be toxic to certain micro-organisms as are quinones (Goodman et al, 1967). Phenols and quinones have been detected in soil and humic acids (Whitehead, 1964; Stevenson, 1967; Schnitzer and Riffaldi, 1972; Mathur, 1972) but their significance in soil microbiology is unclear. Grabbe (1968) indicated that, whereas phenolic compounds inhibit growth of a number of micro-organisms

by uncoupling oxidative phosphorylation, the effect on fungi that synthesise laccase or phenols is minimal. Since compost does not appear to contain toxic levels of free phenols, the possibility exists that mycelial growth of A.bisporus results in the release of phenolic materials from substrate degradation. Laccase secreted during spawn run (Turner et al, 1975) might act as a protecting mechanism for the mushroom by oxidising such phenols, the products of reaction then inhibiting other micro-organisms.

The effect of changes in C:N ratios on compost specificity was considered because of the importance of nitrogen availability in the degradation of complex organic molecules (Garrett, 1963). Recently a high C:N ratio and a low nitrogen supply has been implicated in restricting fungal growth in wood (Levi and Cowling, 1969). Lowering the C:N ratio of oat straw by adding nitrogen increased colonisation by Rhizoctonia solani (Papavizas and Davey, 1961). During the present study changing the C:N ratio of culture media containing soluble nutrients did not affect fungal growth as seriously as manipulating media containing complex carbon sources. When carboxymethylcellulose was supplied as substrate only white rot fungi were able to grow and synthesise enzymes at high C:N ratios. This is in general agreement with the observations on Polyporus versicolor grown on cellulose (Levi and Cowling, 1969). Further observations on substrate utilisation suggested that

A.bisporus was able to produce a wider range of enzymes to degrade complex polymers than other compost inhabiting saprophytic fungi. Although mushroom compost has a total C:N ratio of 16-20:1 (Burrows, 1951; Gerrits et al, 1965) it might have a much higher "available C:N ratio". In this way it might be envisaged that polysaccharide degrading enzymes of fungi other than white rot species would be reduced or prevented, this restricting their growth in compost.

Information on the continued growth of A.bisporus at high C:N ratios was obtained by examining the composition of mycelium grown under high and low C:N ratios. Mycelium grown at high C:N ratios possessed a higher lipid content, but lower quantities of amino acid, proteins, nucleic acids and residual cell wall materials than mycelium grown under low C:N ratios. However, a greater proportion of total nitrogen was bound into nucleic acids at higher C:N ratios suggesting preferential allocation of nitrogen to areas of high metabolic activity (Levi and Cowling, 1969). Under growth limiting conditions e.g. high C:N ratios, there may be extremely efficient conversion of substrates into cell components (Pirt, 1969). Nutritionally hostile conditions induce certain micro-organisms to degrade their proteins to provide amino acids within the cell (Pardee, 1961) thus allowing enzyme synthesis with no net protein gain (Mandelstam and Halvorson, 1960). This could explain the continuous synthesis of enzymes

by white rot fungi in media of high C:N ratios (Levi, Merril and Cowling, 1968). Energy for enzyme synthesis under such conditions may be achieved through degradation of internal structural components or cellular reserves such as glycogen, sugar alcohols or polyhydroxybutyrate (Madelin 1956, 1960; Gray and Williams, 1971b). It is interesting to speculate that the extracellular oxalic acid secreted by mushroom hyphae might act as a reserve energy source.

Since one of the objects of composting is to produce a substrate nutritionally hostile to all micro-organisms except A.bisporus it is necessary to explain those characteristics of the mushroom which enable it to colonise this medium. It is known that inoculum potential of fungi, i.e. the energy for growth at the mycelium-substrate interface (Garrett, 1956), may be responsible for their successful growth in a particular substrate (Buller, 1931; Blair, 1943; Garrett, 1953, 1963; Dimond and Horsefall, 1960). Most fungi examined in this study appeared to be killed on exposure to elevated temperatures, consequently peak heating should successfully reduce their inoculum density in compost. This was previously suggested by Lambert and Ayers (1957), Wuest, Baker and Conway (1970) and Wuest and Moore (1972). When considering the effects of inoculum potential on growth of fungi in compost it was observed that only A.bisporus, Ch.olivaceum and C.fimentarius were able to colonise

it from grain spawn. When inoculum potential was reduced the growth of all fungi except A.bisporus was inhibited. Thus growth of A.bisporus is independent of inoculum potential within the range tested, whereas that of Ch.olivaceum and C.fimentarius appeared to be dependent. Since residual ammonia, under composting, or insufficient oxygen during phase-2 often result in the appearance of Ch.olivaceum or C.fimentarius (Atkins, 1974) it is probable that these fungi survive peak heat as spores. Failure to isolate these fungi during this study might be associated with failure of spores to germinate rather than the loss of their viability. Lack of growth of these fungi in well prepared compost may indicate low inoculum potentials, absence of a germination stimulus, or the involvement of other inhibitory mechanisms discussed above.

Factors influencing competitive saprophytic ability in soil micro-organisms have been discussed previously (Brian, 1960). The later observations of Ko and Lockwood (1970) suggest that the ability of fungal mycelium to withstand microbial leaching might also influence competitive saprophytic ability. Since compost is selective for A.bisporus it presumably contains no inhibitory materials which might prevent or reduce rate of mushroom mycelial growth. Presumably any antibiotic produced by A.bisporus reduces any inhibitory effects of bacteria growing

adjacent to mushroom mycelium as well as reducing competition for nutrients. An important factor in saprophytic survival of micro-organisms is the nature and availability of nutrient substrates. The nature of the substrates degraded by A.bisporus has been the subject for much research.

In their early work on the nature of changes occurring during composting Waksman and Gerretson (1931) suggested that a build-up of micro-organisms occurred during the process, this was also proposed by Gerrits et al (1965). Hayes (1972) considered that the function of this synthesised microbial biomass may be to provide growth factors for the mushroom. Despite the recognition of such an accumulation of micro-organisms few attempts have been made to determine their importance in mushroom nutrition. Waksman and Nissen (1932), Gerrits et al (1965), Grabbe (1968), Park (1971) and Stanek (1968, 1972) have suggested that essential nutrients, amino acids, peptides, proteins and vitamins may be synthesised during composting. Stanek (1972) has proposed that extracellular bacterial polysaccharides provide a suitable nutrient source for the mushroom in vitro. Gerrits et al (1965) assumed, without supporting evidence, that A.bisporus could utilise compost micro-organisms and their metabolic waste products as nutrient sources.

One of the first microbiological studies of compost using isolation techniques and microscopy

revealed that a succession of micro-organisms occurred in the substrate during decomposition (Waksman and Gerretson, 1931). Incident light microscopy and electron microscopy have subsequently been used to study actinomycetes in soil(Szabo, Marton and Portai, 1964) while scanning electron microscopy has been used to examine other micro-organisms, including bacteria and fungi, in soil (Gray, 1967; Mayfield et al, 1972). Light (Corbett and Levy, 1963) and electron microscopy (Levi and Preston, 1965) have been used in studies of "soft rot" fungi on decaying wood and related to chemical and biochemical analyses. In the present study light and scanning electron microscopy of wheat straw during composting has been supported by chemical extraction techniques at various stages in the decomposition process.

Cellulose containing phloem cells appeared to be rapidly degraded during phase-1 of composting but lignified cell walls remained relatively intact. However, the techniques used were not sufficiently sensitive to detect changes in cellulose and lignin in cell walls so these observations must be interpreted with caution. Changes occurring in the structural components of straw cell walls requires more thorough analysis since the details of utilisation of lignin, cellulose and hemicellulose in compost preparation and the subsequent contribution of these materials to the

life cycle of A.bisporus is unclear. An approach adopted by Santra and Nandi (1975) for studying the decay of wood by Mimusops elengi involving a histological examination supported by microchemistry and histochemistry might be adopted in future. This could be linked with studies using ^{14}C-labelled lignin or cellulose to follow rates of degradation of these materials (Mayaudon and Simonart, 1960) and the fate of the degraded components in compost.

Concurrent with the degradation of plant materials during composting there was an accumulation of dark material on straw which appeared to be mainly composed of microbial remains. During mushroom growth this black surface layer is removed suggesting that it is utilised as a nutrient source. This was previously postulated by Sinden and Hauser (1953) who assumed the black material to be products of chemical reactions at elevated temperatures.

Degradation of numerous other substrates by the mushroom has been examined (Styer, 1930; Treschow, 1944; Bohus, 1959) but it was not until recently (Vincent-Davies, 1971) that A.bisporus was shown to be capable of producing the enzymes necessary to degrade the major structural components of fungal cell walls. The fate and composition of microbial residues in soil has been reviewed (Webley and Jones, 1971) but a similar review of microbial residues in mushroom compost does not exist. Parasitism of lower fungi by basidiomycetes has been recorded (Griffin and Barnett, 1967),

degradation of fungal residues in soil by fungi being pH dependent (Gray and Williams, 1971b). Rapid degradation of bacterial and fungal cells by mixed populations of soil micro-organisms has been demonstrated using ^{14}C-labelled cells (Mayoudon and Simonart, 1963). During the present study there was some indication that quiescent thermophilic bacteria and fungi might provide a nutrient source for A.bisporus. Degradation of living thermophilic micro-organisms in vitro and reduced microbial numbers in compost occurred with mushroom mycelial growth. Such a decline in numbers of micro-organisms has been recorded previously but its significance was not considered (Fordyce, 1970). The mushroom was shown to produce β 1-3 glucanase but not chitinase in spite of the claim that A.bisporus can degrade chitin (Vincent-Davies, 1971). Lysis of bacteria by fungi is a subject receiving little attention though Hash (1963) using purified enzymes from Chalariopsis sp. from soil succeeded in lysing cells of Staphylococcus aureus. Because of the large thermophilic bacterial population in compost (Chanter and Spencer, 1974) the significance of microbial cellular residues to mushroom growth should be examined in more detail.

The contribution made by micro-organisms to the chemical composition of composted materials has seldom been recognised. Norman (1942) has suggested that the fraction of soil organic matter designated as

hemicellulose in the "proximate" system of analysis (Waksman and Stevens, 1930) probably represents a measure of microbially synthesised polyuronides. Treschow (1944) criticised Waksman and McGrath (1931) and Waksman and Nissen (1932) for not recognising the part played by mushroom mycelium in data obtained by "proximate analysis". Similarly, insoluble nitrogen in compost is assumed to be protein in nature (Waksman and Iyer, 1932; Gerrits et al, 1965; Hayes, 1972), the importance of other possible sources, e.g. microbial cell walls, or humic acid bound amino acids or amino sugars (Bremner, 1952, 1955; Bondietti et al 1972) has not been considered.

Because of the colour change in compost colonised by A.bisporus it was not unreasonable to assume that the humic acid fraction was being degraded. After acid hydrolysis of alkali humic acid extracts it became evident that the attached polysaccharides had decreased and little quantitative change in humic acid fractions occurred. Similarly the acetone insoluble polysaccharide from the fulvic acid fraction appeared to be readily degraded. Chromatographic analysis of component monomers indicated that polysaccharides were primarily microbial in origin. Sugars identified in hydrolysates of compost or isolated compost polysaccharides included glucose, galactose, arabinose, mannose, xylose, ribose, fucose and rhamnose. Whilst plant and animal polysaccharides normally consist of one to three, and

rarely four or five, different monomer units (Martin, 1971) polysaccharide fractions isolated from soil normally contain more than ten major sugar units and many others in smaller quantities (Forsyth, 1954; Swincer et al, 1969; Martin, 1971). The wide range of sugar units detected from compost suggest the involvement of polysaccharides which are microbial in origin. Low levels of xylose, as detected in this study, suggest that hemicellulose is not a major component of the extracted polysaccharide material.

Whilst recognising the limitations imposed by the analytical techniques used in this study it is possible to postulate that microbial polysaccharides constitute a major fraction of compost and could provide nutrients for A.bisporus during active growth of mycelium in compost. Bacterial extracellular polysaccharides are normally produced abundantly under conditions of limiting nitrogen (Wilkinson et al, 1955) particularly by cellulose decomposing strains (Russell, 1973) a condition encountered in compost. Addition of sugar or molasses to raw materials during composting (Hayes and Randle, 1968), or skimmed milk powder before the preparation of express substrates (Smith pers.comm) has resulted in increased yields from supplemented composts. This was interpreted as a conservation of cellulose during composting for subsequent utilisation by A.bisporus (Hayes and Randle 1968). Another interpretation is that the low nitrogen levels combined with high readily available carbon results in the synthesis of microbial

polysaccharides, these then acting as substrates for A.bisporus. The mushroom is able to utilise such materials in vitro four to seven times better than glucose (Stanek, 1972) an observation confirmed in the present study.

Microbial polysaccharide has not previously been examined in compost though polysaccharide constituents of soil fulvic and humic acids have been well documented (Mehta, et al, 1961; Swincer et al, 1968; Tan and Clark, 1968; Martin, 1971). The extraction procedures used in the present study removed polysaccharide materials equivalent to 40% dry weight peak heated compost. The exact proportion of microbial polysaccharide remains to be determined but preliminary results suggest that this could constitute a very significant proportion of the whole.

Synthesis of polysaccharides in mushroom mycelium should not be overlooked. In their early work Waksman and McGrath (1931) suggested that the mushroom contained the following as major components: hot water soluble organic matter 42%; hemicellulose, 14%; cellulose, 5%. More recent studies (Vincent-Davies, 1971; Michaelenko, Hohl and Rast, 1976) indicate the hyphal wall to be composed of an inner layer of chitin microfibrils, in a β-glucan matrix also containing protein, and an outer layer of KOH-soluble αglucan; a layer of β-glucan mucilage may also be present on the outer surface. Glucans

represent about 41% mycelium dry weight, while protein accounts for 16%.

In the commercial situation there is a considerable synthesis of mycelium during spawn run. In many instances the density of this mycelium decreases during cropping. Unfortunately it has not been possible to date to accurately quantify such growth but after the third flush it is often apparent that a considerable reduction in the amount of mycelium has occurred. Often only thicker strands of mycelium can be observed at this stage. This change and its possible significance appear to have been largely overlooked in the past. Certain fungi, notably basidiomycetes, grown under limiting nutrient conditions may degrade older fractions of their own mycelium to provide nitrogen for sporophore development (Morten and Broadbent, 1955; Merrill and Cowling, 1966; Levi, Merrill and Cowling, 1968). After basidiocarp initiation in Coprinus lagopus vegetative cells lose their nutrient reserves and vacuolate suggesting the translocation of nutrients to developing sporophores (Madelin, 1956; 1960; Burnett, 1970). Hein (1930) observed that the first sporophores on mushroom beds initiate a flow of nutrients in their direction and away from other primordia. In their extensive biochemical analysis of the development of carpophores in Schyzophyllum commune Wessels (1965) & Niederpruem and Wessels (1969) confirmed the translocation

hypothesis and suggested the kind and location of materials involved. In this fungus over 80% of wall carbohydrate is glucan. During the phase of pileus formation there is a marked degradation of residual glucans and subsequent translocation of glucose to developing sporophores. Thus sporophore development in some basidiomycetes is linked with the depletion of cytoplasmic and cell wall storage reserves which are incorporated into developing sporophores. It is possible that carpophore development in A.bisporus is similar to that discussed for S.commune. Recent observations by Hammond and Nicholls (1976) would tend to support this suggestion. Whether sufficient nutrients exist in the mycelium to provide totally for the development of large numbers of sporophores produced by A.bisporus under commercial conditions is unknown. There may be an additional substrate requirement, possibly residual cellulose in the straw etc. Increased cellulase production in compost is a feature associated with sporophore enlargement (Turner et al, 1975). Sugars derived from cellulose or other polysaccharides could be incorporated directly into the sporophore or used to replace depleted storage reserves (Hammond and Nicholls, 1976). Cowling (1961) and Petterson et al (1963), demonstrated the degradation of amorphous and crystaline cellulose by Polyporus versicolor and suggested that certain white rot fungi have the

capacity to produce C_1, the prehydrolytic enzyme, together with C_x (β 1-4 glucanase). The failure to detect C_1 enzyme from A.bisporus in the present study might reflect the use of unfavourable cultural conditions (Norkrans, 1967). Gascoigne and Gascoigne (1960) suggested that for both bacteria and fungi cellulolytic enzymes were highly localised but evidence exists to suggest that hymenomycete cellulases may act at considerable distances from the mycelium (Cowling 1961). In wood depolymerisation of cellulose by micro-organisms is slow, each constituent being depolymerised as rapidly as degradation products are metabolised. An induction-repression mechanism might be operative in this situation since low levels of free glucose repress cellulase synthesis in some micro-organisms (Norkrans, 1967). Lignin degradation is supposed to precede cellulose decomposition in decaying plant material (Norkrans, 1967), which might explain the delayed cellulase production during the growth cycle of A.bisporus (Turner et al, 1975).

The results obtained during the present study on synthesis and utilisation of bacterial polysaccharides in compost highlight the need for further examination. An assay of compost for the enzymes involved in polysaccharide degradation might be supplemented by a study of decomposition of ^{14}C labelled microbial polysaccharide added to compost.

Few studies have been made on the decomposition in soil of ^{14}C labelled extracellular microbial polysaccharides. However, Oades and Wagner (1971) and Martin et al (1974) have examined degradation of labelled polysaccharide in soil and claim that the rate of degradation is variable but in most cases is relatively slow. Resistance of microbial polysaccharides to degradation in soil has previously been recognised (Norman, 1942) and may result from attachment to decomposing plant matter (Griffiths and Burns, 1972) or humic materials (Martin, 1971). It is interesting to speculate that the laccase secreted into compost during spawn run (Turner et al, 1975) might be involved in enzymically modifying humic materials in such complexes to release attached polysaccharide. The stabilisation of protein in protein-humic acid complexes may be due to an inhibition of proteolytic enzymes (Ladd and Brisbane, 1967). A similar situation may exist for polysaccharide-humic acid complexes to inhibit polysaccharide degrading enzymes in compost.

Humic materials from soil may be derived in a number of ways (Felbeck, 1971), one or more of these may account for humic acid synthesis in compost. These materials are relatively resistant to rapid microbial degradation (Mathur and Paul, 1967; Grabbe, 1972) which could account for the small decreases

observed in compost during this study.

The nitrogen-rich lignin-humus complex (Burrows, 1951; Gerritts et al, 1965) has been suggested as an important source of nitrogen for the mushroom. Small changes in the humic acid content of compost during spawn run and cropping, as recorded in this study, could represent the utilisation of this nitrogen, which is assumed to be protein in nature (Waksman and Iyer, 1932; Gerrits et al, 1965). This nitrogen containing portion is probably strongly bound to the lignin-humic material and is resistant to degradation by most micro-organisms. Nothing is known of the mechanism by which A.bisporus removes nitrogen from this type of complex.

Thus it appears that the mushroom occupies an environment hostile to most micro-organisms because they are unable to utilise the complex residues which remain after composting. The results of this study suggest that this is the primary reason for specificity in compost. Selective growth of A.bisporus in compost is thus dependent on the ability to produce a wide range of enzymes, over wide C:N ratios, for substrate utilisation and is favoured by the production of volatile materials inhibitory to other micro-organisms. Mushroom compost, whilst being low in freely available nutrients, is thus rich in substrates available to A.bisporus. It is suggested that one substrate of major importance is extracellular

microbial polysaccharide which is used during spawn growth.

The importance of extracellular polysaccharides is of particular interest in the preparation of express substrates, a practice likely to increase in future because of potential saving in raw materials, labour, time and costs. Supplementation of raw materials relatively low in nitrogen with readily degradable carbon sources, possibly industrial waste products e.g. molasses, sugar beet pulp, skimmed milk powder, should result in an increase in microbial biomass very much in excess of that obtained during the restricted phase-1 procedures involved in this technique. After spawn run these composts could be supplemented with nitrogen containing substances, e.g. cotton seed or soya meal, or lipids to increase yields of sporophores (Laborde and Delmas, 1969; Huhnke, 1972). In composts prepared by the 2-phase method (Sinden and Hauser, 1953) the addition of refined or crude oils, or lipids as supplements has resulted in marked increases in yields of sporophores (Schisler and Sinden, 1966; Schisler, 1967). Recently Schisler and Patton (1970) demonstrated that linoleic acid could account for stimulation in yields resulting from vegetable oil supplementation of compost, and that ethyl linoleate produced parallel effects. Later Holtz and Schisler (1972) suggested that linoleic acid was utilised as a source of acetate units for synthesis of sporophore lipids by A.bisporus.

Wardle and Schisler (1969) recorded increased mycelial growth in media supplemented with lipids but yield increases in the commercial situation probably also reflect increased production of sporophore initials.

Although detailed analysis of the fate of straw polymers are not available it is very probable that cellulose and hemicellulose in express substrates is preserved because of the reduction in composting time. Thus the possibility for recycling express substrate compost exists particularly if the nutritional value of microbial detritus is thoroughly investigated. The problem of reutilising express substrates might be resolved by investigating mechanisms for re-establishing microbial populations to confer specificity and provide a nutrient source for mushroom growth.

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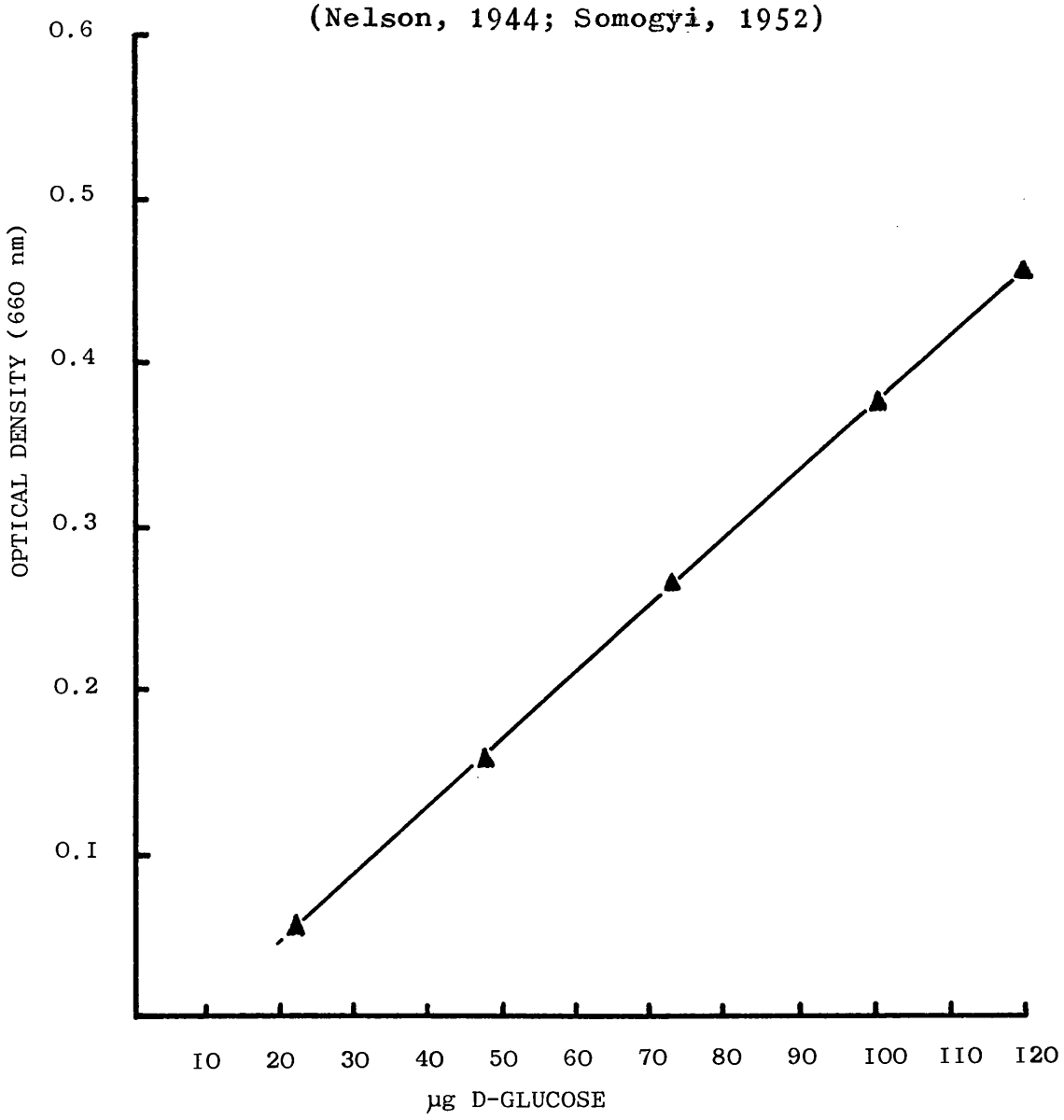
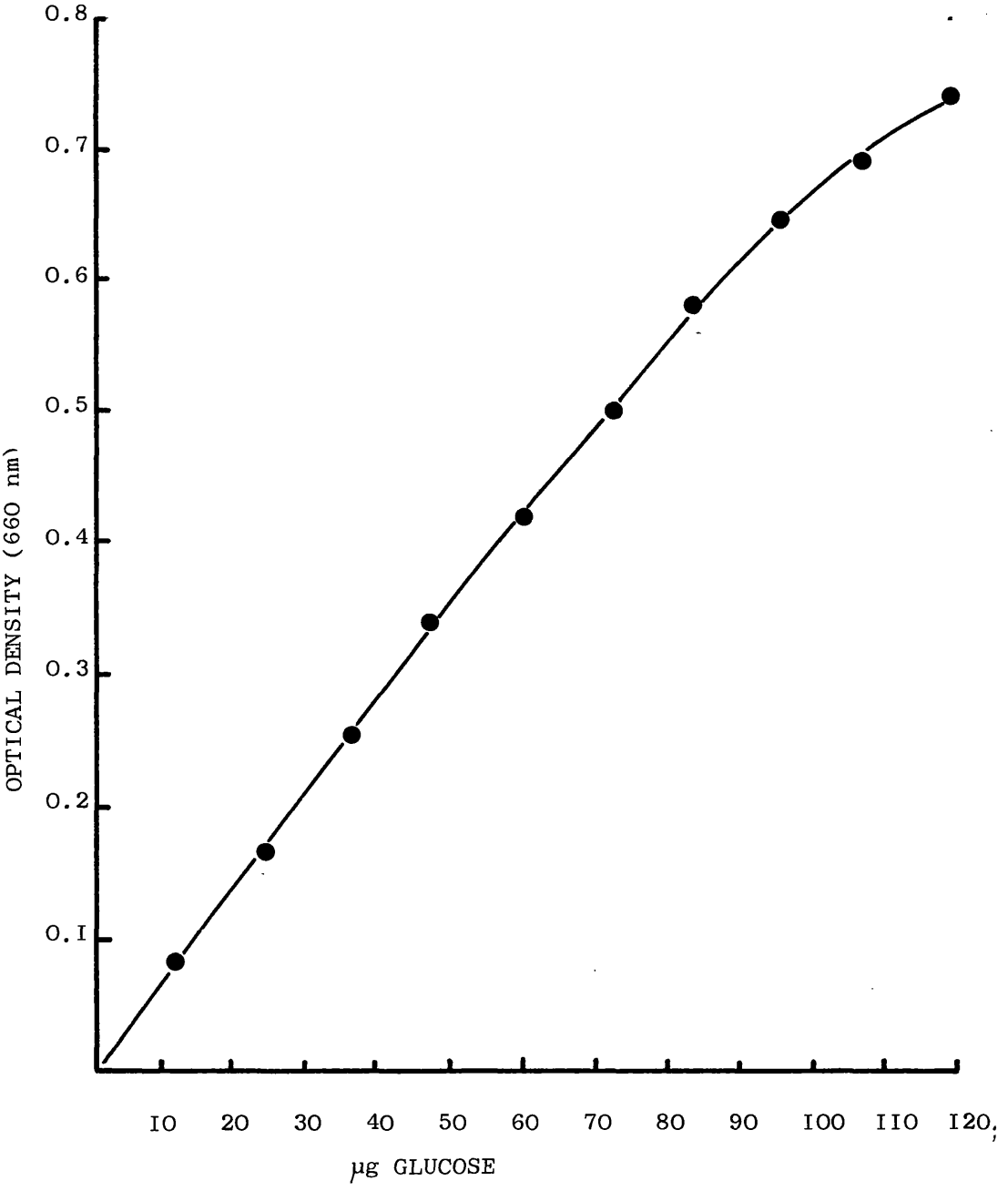
APPENDIX 1

Standard Line for glucose or reducing sugar estimation

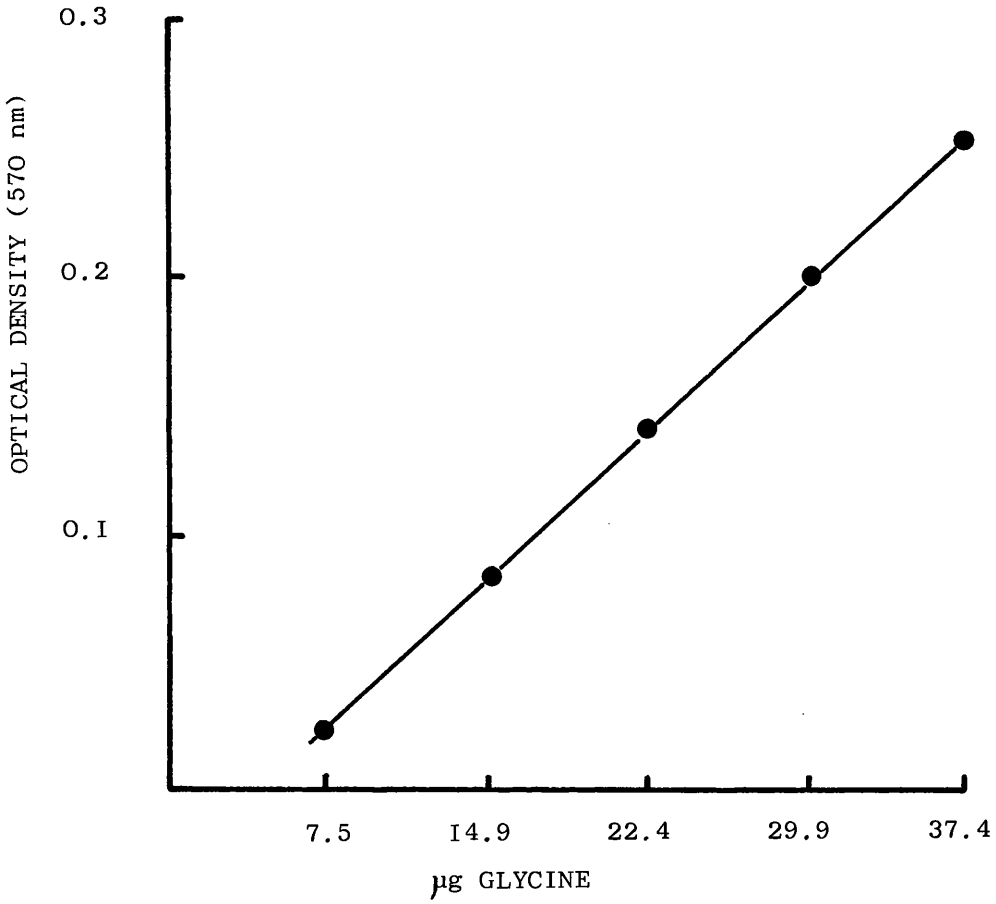
(Nelson, 1944; Somogyi, 1952)



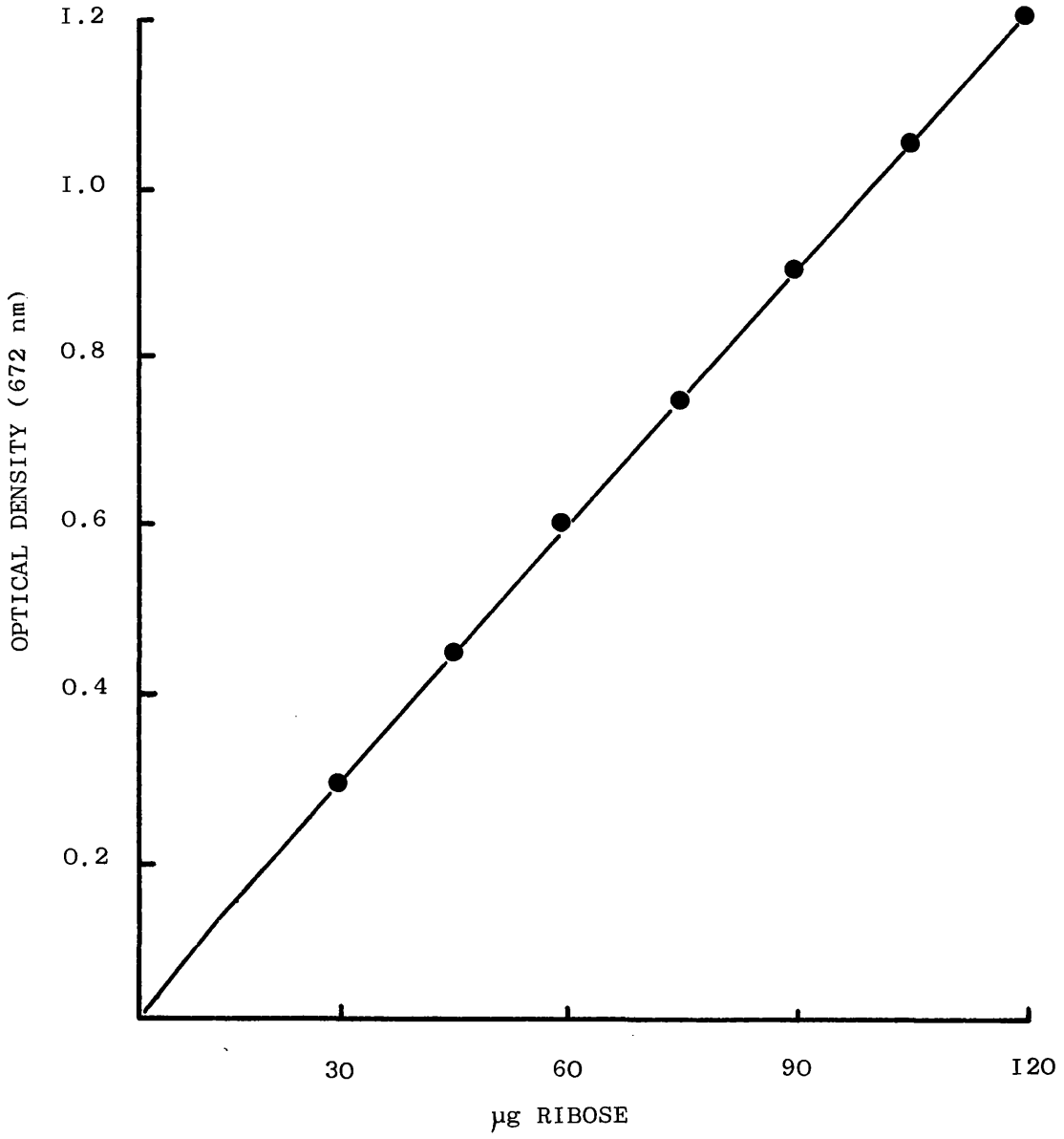
Standard line for total carbohydrate estimation with anthrone reagent (Morris 1948)



Standard line for ∝ amino acid estimation
(Lee and Takahashi, 1966)



Standard line for pentose estimation
(Herbert et al, 1971)



APPENDIX 2

Table 5a

Effect of volatile metabolites from peak heated compost on numbers of compost micro-organisms*

Organisms/g dry wt.		Plates incubated over peak heated compost	Plates incubated over sterile compost
bacteria	($x10^8$)	11.5	47.4
actinomycetes	($x10^7$)	18.8	17.7
fungi	($x10^4$)	16.0	17.3

*sealed plates

Table 33a

Effect of volatile metabolites from A.bisporus on development of compost micro-organisms*

Organism/g dry wt.		Substrate: Sterile compost	Peak heated compost	Spawn run compost	Sterile compost with mushroom	L.S.D. (p=.05)
bacteria	($x10^8$)	1.7	11.5	0.4	43.6	13.0
actinomycetes	($x10^8$)	2.4	18.8	0.2	1.4	17.0
fungi	($x10^4$)	17.3	16.0	11.4	17.4	NS

*sealed plates

Table 34a

Effect of volatile metabolites from mushroom on growth of saprophytic fungi (plates sealed)

Test organism	Percentage growth*				
	Peak heated compost	Spawned compost (14 days)	Spawned compost (21 days)	Sterile compost + mycelium (21 days)	Incubation time (days)
V. malthousei	- 7.4	-33.2	-14.8	-22.3	7**
Penicillium sp.	- 8.4	- 9.8	-38.0	-21.2	6
Oedocephalum sp.	-26.5	-52.0	-80.3	-12.0	3
C.tropica.	0	-12.0	-35.3	0	3
Chaetomium olivaceum	-12.2	-60.0	-70.0	-28.7	4
Absidia sp.	-30.0	-54.8	-45.3	- 9.6	4
Phycomycete sp.	-21.6	-29.7	-20.3	-18.9	4
D.dendroides.	-34.5	-50.0	-51.0	-51.0	7**
C.albicans.	0	- 4.8	-28.7	-22.9	7**
T.roseum.	+13.5	-37.4	-35.6	-35.6	7**
Coprinus.	0	-78.6	-51.2	0	3

* diameter of colony exposed to volatiles as a percentage of growth in normal atmosphere

** signs of anaerobic growth

Table 35a

Relation between production of volatile metabolites and spore germination in sealed plates

Test organisms		Substrates			
		sterile compost	sterile compost + mycelium	spawn run compost(14 days)	peak heated compost
D.dendroides	%*	99.0	98.6	1.3	100
	g.t.l.**	0.40	0.6	0	0.7
T.roseum	%	99.0	99.6	84.3	100
	g.t.l.	0.68	0.50	0.05	0.60
Fusidium sp.	%	99.3	100	26.0	99.6
	g.t.l.	0.2	0.79	0.05	0.61
Penicillium sp.	%	99.3	98.0	48.6	93.0
	g.t.l.	1.16	0.56	0.04	1.07
S.stemonitis	%	99.3	97.6	3.3	100
	g.t.l.	0.92	0.78	0	0.61

%* = percentage germination (28 hr.)

g.t.l.** = germ tube length (mm).

APPENDIX 3

Solute	Solvent (R.f.)*	
	acetic acid: chloroform	ethyl acetate: benzene
catechol	0.34	0.53
shikimic acid	0	0
caffeic acid	0.17	0.07
vanillic acid	0.52	0.14
ferulic acid	0.52	0.12
chlorogenic acid	0	0
hydroxycinnamic acid	0.65	0.83
syringaldehyde	0.55	0.39
pyrogallol	0.13	0.35
vanillin	0.58	0.49
p-hydroxybenzaldehyde	0.40	0.50
verataldehyde	0.70	0.84
4-methyl catechol	0.43	0.70
α -naphthol	0.69	0.75
β -methyl ketone	-	0.90

* R.f. values obtained after thin layer chromatography. Spots visualised using U.V. light and diazotised sulphanilic acid.